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Volume LXXI Number 2

THE BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

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OCTOBER, 1936

Printed and Issued by
LANCASTER PRESS, Inc.
PRINCE & LEMON STS.
LANCASTER, PA.

THE BIOLOGICAL BULLETIN

THE BIOLOGICAL BULLETIN is issued six times a year. Single numbers, $1.75. Subscription per volume (3 numbers), $4.50.

Subscriptions and other matter should be addressed to the Biological Bulletin, Prince and Lemon Streets, Lancaster, Pa. Agent for Great Britain: Wheldon & Wesley, Limited, 2, 3 and 4 Arthur Street, New Oxford Street, London, W.C. 2.

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Entered October 10, 1902, at Lancaster, Pa., as second-class matter under Act of Congress of July 16, 1894.

Vol. LXXI, No. 2 October, 1936

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BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

THE REACTIVATION BY CUTTING OF SEVERED MELANOPHORE NERVES IN THE DOG-FISH MUSTELUS

G. H. PARKER

(*From the Woods Hole Oceanographic Institution, Woods Hole, Mass.*[1])

When the melanophore nerves in many lower vertebrates are cut, the areas of skin thus denervated quickly turn dark and remain so for some considerable time. This darkening results from a dispersion of pigment in the melanophores of the denervated areas. Such occurrences were apparently first noted in the common chameleon in 1852 by Brücke, whose observations were confirmed on South African species by Hogben and Mirvish (1928) and by Zoond and Eyre (1934). In bony fishes similar darkenings of the skin as a result of nerve cutting have been recorded by Pouchet (1876), von Frisch (1911), Wyman (1924), Hewer (1927), Giersberg (1930), and Smith (1931), to mention only a few of the more recent workers. Brücke expressed the opinion that melanophores were related to their nerves as muscle-fibers are to motor nerves and that, since the cutting of motor nerves induces paralysis in the neuro-muscular system, the same should be true of melanophore nerves and their effectors. He therefore advanced the idea that severed melanophore nerves with their expanded color-cells are in a state of paralysis. This interpretation of the condition of these parts has been accepted by almost all investigators in this field, even the most recent (Keller, 1895; von Frisch, 1912*a*; Spaeth, 1916; Giersberg, 1930; Zoond and Eyre, 1934; Sand, 1935). In opposition to this view I have advanced the idea that when a melanophore nerve is cut the injury thus inflicted throws the nerve into a state of unusual but temporary activity which may last, however, even for some days. This opinion is based chiefly upon observations on the caudal bands of the common killifish, *Fundulus heteroclitus* (Parker, 1934).

If a dark band is produced by the usual cut in the tail of a pale *Fundulus*, this band, as is well known, will fade out in the course of a few

[1] Contribution No. 113.

days and become almost if not quite indistinguishable in tint from that of the rest of the fish. If now a second cut is made slightly distal to the first one and within the limits of the original band, a second somewhat smaller band will form between the new cut and the edge of the fin. This second band may be induced at any period after the first band has faded sufficiently and before the degeneration of the melanophore nerves has set in, a disturbance which begins to appear some five days after the initial cut has been made. Any time during these first five days on application of a cold block to such a dark band the part of the band distal to the block may be temporarily obliterated. Or if a cut is made proximal to a block, in a tail in which there is no band, a band will form from the cut to the block but not beyond it, though a cut distal to the block will induce a second band from the new cut to the edge of the tail. These several conditions show that the dark band that immediately results from a cut in the tail of *Fundulus* is not due to the paralysis of the detached portion of the neuro-melanophore system as maintained by almost all previous workers (paralysis hypothesis) nor to the blocking of impulses as suggested by Zoond and Eyre (1934), but is occasioned by the local irritation of the dispersing melanophore nerve-fibers, an irritation that may last for some days (superactivity hypothesis).

The situation thus briefly outlined is based upon experiments carried out on *Fundulus*. During the past summer an opportunity was offered at the Woods Hole Oceanographic Institution to apply similar tests to elasmobranchs. The smooth dogfish, *Mustelus canis*, possesses only one set of melanophore nerves, namely, those concerned with pigment concentration. Consequently when a cut is made in the pectoral fin of this fish, a pale band appears in this organ (Parker and Porter, 1934). Such bands disappear in the course of time as the dark bands in *Fundulus* do. If after such a band in a smooth dogfish has faded out (Fig. 1), a second

EXPLANATION OF FIGURES

FIG. 1. Right pectoral fin of *Mustelus* seen from above. The initial cut (lower) was followed by the formation of a pale band extending from the cut to the edge of the fin. After this band had darkened as appears in the photograph a second cut was made (upper) proximal to the first one. This second cut produced no observable paleness in the fin.

FIG. 2. Left pectoral fin of *Mustelus* seen from above. The initial cut (upper) induced the formation of a pale band extending from the cut to the edge of the fin. After this band had darkened (Fig. 1), a second cut (lower) was made distal to the first one. This second cut produced the faint splotchy pale area which extends from the region of the cut to the edge of the fin.

FIG. 3. Right pectoral fin of *Mustelus* seen from above. The pale band from the initial cut had darkened before the second cut distal to the first one was made. This second cut excited the formation of a very distinct distal splotch within the area of the former band.

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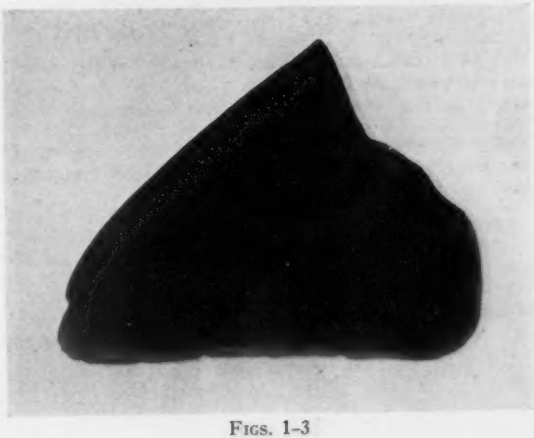

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FIGS. 1–3

cut is made within the limits of the first one and distal to it, a second fainter band commonly much limited in extent will become visible within the area of the first (Figs. 2 and 3). In *Mustelus* both the primary and the secondary bands are much less regular than in *Fundulus*. This is probably due to the less regular course of the melanophore nerves in the dogfish than in the killifish and to the fact that in the dogfish the one nervous reaction, concentration, is overcome by a humoral response, dispersion, dependent upon a diffuse pituitary neurohumor in the blood. The important point in this discussion, however, is the fact that in *Mustelus* a pale band after it has become dark can be revived by the recutting of its nerves showing that these nerves were not paralyzed by the first cut but remained functional and that the blanching of the band is the result of some form of irritation in the cut and not of paralysis nor of the blocking of impulses. These observations then are in accord with those already reported for *Fundulus* and in this respect support the superactivity hypothesis. Attempts were also made to produce bands in the pectoral fins of the skate *Raja erinacea*, which shows well marked color-changes (Parker, 1933), but the cutting of nerves in the pectoral fins of this fish was not followed by the formation of bands to any observable extent. In this respect *Raja erinacea* is like *Squalus acanthias* in which fin bands are scarcely if at all elicitable (Parker, 1936).

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PHYSIOLOGY OF THE MELANOPHORE SYSTEM IN THE CATFISH, AMEIURUS[1]

A. A. ABRAMOWITZ

(From the Biological Laboratories, Harvard University, and the Marine Biological Laboratory, Woods Hole, Massachusetts)

I. Introduction

The color changes of the catfish, *Ameiurus nebulosus* (Leseur), have been studied recently by Parker (1934*a*) in relation to neurohumors. This fish can be made to assume a yellowish-green color in an illuminated white vessel within three hours, and becomes remarkably dark in an illuminated black vessel within one hour. Such changes are known to be due to the concentration or dispersion of the pigment contained within pigment cells or chromatophores, and are brought about not only through stimulation of chromatophore nerves but also through the coöperation of hormones. One of these hormones is that of the pituitary gland which, according to Parker, is secondary to the dominant nervous system. Its effect on the melanophore pigment of the catfish is dispersion, an effect characteristic of intermedin, the hormone of the intermediate pituitary (Zondek, 1935). Another hormone which this paper will show to be in operation in special states is similar in effect to adrenalin. The existence of such a substance has already been advocated by Smith (1931) to be responsible for the pallor of denervated regions of melanophores in *Phoxinus*. In the year following Smith's publication, however, a rather novel hypothesis (the neurohumoral theory, Parker, 1932) was advanced to explain the behavior of denervated melanophores. Thus, two mechanisms have been advanced to account for the reactivity of denervated chromatophores. It was therefore decided to reinvestigate the catfish from both of these points of view, and to determine the factors and mechanisms which would account for all of the demonstrable chromatic responses of this fish.

[1] This work was carried on under the direction of Professor G. H. Parker and, with the exception of the section on the pituitary gland, was submitted as part of a thesis presented for the doctorate degree, Harvard University, 1935. To Professor Parker I am greatly indebted for much advice and innumerable kindnesses. Acknowledgments for research facilities are due Dr. M. H. Jacobs, Director of the Marine Biological Laboratory, Woods Hole, where these investigations were carried on in the summers of 1933 and 1934.

II. Material and Methods

The catfish, *Ameiurus*, was used for the experiments here being reported. Specimens were obtained in Cambridge during winter months from local dealers, and during summer months from neighboring fresh water ponds in the vicinity of the Marine Biological Laboratory, Woods Hole. In the laboratory they were kept in large tanks of running water. When used for experimental purposes the animals were maintained in white porcelain dishes or in large glass vessels the outsides of which had been painted black. Such vessels have been used for many years in this laboratory for the induction of desired chromatic responses. Ordinary Mazda lamps provided illumination of the white and black backgrounds, both being illuminated with lamps of equal light intensities. Photographic darkrooms were employed in the study of the behavior of animals to light or darkness, and, unless otherwise stated, all experiments were carried out at room temperature.

III. Experiments

A. The Relation of Melanophores to the Nervous System

1. *Pigment Motor Pathways.*—The dominance of the nervous system over the pigmentary responses of teleosts has never been threatened seriously by any other agency. The general arrangement of the pigmentary nerve tracts has been determined for many fishes, but for practical reasons this was omitted in the catfish. It is probable that the catfish is not different in this respect from *Phoxinus* and other European fishes (von Frisch, 1911), the English dab (Hewer, 1926*b*), the scorpion fish (Smith and Smith, 1934), and *Fundulus* (Spaeth, 1913; Abramowitz).[2] It may be stated with a good measure of accuracy that the general arrangement of the pigmentary nervous tracts is more or less constant among teleosts. Pigment motor fibers extend from the brain for some distance through the spinal cord. At a definite point, which may vary slightly for different fishes, the fibers leave the spinal cord and enter the sympathetic chains from whence they are distributed to all portions of the integument mainly by way of the segmental spinal nerves. When any part of this system is disrupted, a darkening of the skin to which the cut nerves are distributed is apparent. There seems to be a no more universal response of teleost melanophores than that of expansion following a denervating operation. The use of denervating operations in the elucidation of normal pigmentary responses is, as Parker's work has shown, well worth while, for denervated cells may be studied side by side with normal cells in a small area of the integument.

[2] Unpublished work: "The Physiology of the Innervation of Fish Melanophores," doctorate thesis, Harvard University.

The most convenient region in the body of the catfish in which the denervating operations may be performed is the caudal fin. This fin is supported by some fifteen rays. Parker (1934*a*) demonstrated that the main nerves pass out in the rays to the chromatophores of the tail and consequently the cutting of a ray by means of a transverse incision severs the nerve which controls that part of the fin. A dark band extending from the incision to the distal edge of the fin is therefore produced.

2. *Blinding.*—The melanophores in these dark bands (primary caudal bands) are, of course, denervated and therefore incapable of ordinary responses to backgrounds, responses which are mediated by the eyes. That removal of the eyes causes a darkening of the animal is an observation reported repeatedly not only by investigators interested in its chromatic responses but also by those having occasion to use this fish for other purposes. Optic enucleation is much like cutting a peripheral nerve, the main difference superficially being that blinding results in a general effect, whereas destruction of a peripheral nerve produces only a local response. Blinded catfishes naturally lose the capacity to respond to backgrounds, but they do not lose all of their chromatic responses, for they react in a very positive manner to variations of light intensities. This matter, however, will be taken up in a subsequent section.

3. *Denervated Melanophore Responses.*—Just as the melanophores of blinded animals do not lose their capacity to react, the denervated cells of the caudal bands do not suffer loss of the ability to undergo pigment alterations. The early investigators spoke of denervated cells as paralyzed, but Parker has amply demonstrated complete activity of these so-called paralyzed cells. The following account is taken from Parker (1934*a*) and serves to illustrate what has been designated as neurohumoral activity. A transverse cut across one or two fin rays, as has been mentioned, produces a dark band in which the pigment of the melanophores is fully dispersed. This dark band fades if the fish is kept in an illuminated white vessel for three days but suffers no change in tint if the animal is maintained in a black vessel. If the animal which has been kept in a white vessel and whose caudal band in consequence of this treatment has become fully pale is transferred to a black dish, the caudal band darkens at the same time as or slightly later than the remainder of the animal. The fading of a dark band is accomplished, according to Parker, by a concentrating neurohumor which diffuses into the denervated area as a secretion from the intact concentrating nerve terminals of the flanking tail areas. The reverse change is due to the combined effects of the pituitary hormone and of a dispersing neurohumor secreted from the intact dispersing nerve terminals of the adjacent areas. Of these two dispersing agencies, the pituitary hormone is the weaker.

as indicated by the fact that the same neurohumoral responses occur in hypophysectomized catfishes (Parker, 1934*a*).

4. *Degeneration and Regeneration of Radial Nerves.*—As is expected, the melanophores of the caudal band remain functionless as far as ordinary nervous responses are concerned. The neurohumoral experiments of Parker bear evidence to this. Moreover, if a totally dark animal possessing a dark caudal band is stimulated electrically through the roof of the mouth, the animal pales within several minutes but the band remains fully dark at the end of this time. In a similar but totally pale animal an incision made in the tail tissues somewhat anterior to the point of initial transection causes only a dark block between the two

TABLE I

Rate of regeneration of concentrating radial nerves of Ameiurus nebulosus *in millimeters per day.* This table is a sample table of data obtained in the study of chromatophore nerve regeneration. These determinations were made during the months of July and August, and consequently the rate is slightly higher than that which has been given in the text. The average rate computed at various intervals of the year is 0.51 mm. per day.

Length of denervated area	Rate of regeneration in millimeters per day measured at the end of the following days:							
	4.3	9	12	17	22	24.3	30	Average
Fish I..... 16 mm.	0.70		0.50	0.53	0.55		0.43	0.54
Fish II..... 16 mm.	1.15	0.95	0.92	0.75		0.53	0.53	0.80
Fish III..... 14½ mm.	1.0	0.78			0.50		—	0.76
Fish IV..... 17½ mm.	0.93	0.89		0.59	0.55		0.47	0.72
Fish V..... 16½ mm.	0.70		0.50	0.41	0.45	0.54	—	0.52

— = regeneration completed. Average 0.67 mm. per day.

transverse cuts. These standard experiments demonstrate that no central impulses can be transmitted across the cut nerves. Following the initial incision used to produce primary bands, the cut nerves undergo progressive degeneration (Parker, 1934*a*) so that thirteen days later their distal tracts have degenerated completely.

Depending on the temperature and possibly other factors, regeneration of chromatophore nerves begins about two weeks after the initial operation and commences from the central stumps of the cut nerves. Nerve regeneration progresses at the rate of a half-millimeter a day (Table I), as determined by the method described by Abramowitz (1935). Active regenerative growth of nerves, as reflected by the reactivity of melanophores, may be demonstrated by a variety of methods.

If a totally dark animal (operated on three weeks previously) is placed on a white background or stimulated electrically, the typical pallor results and this pallor includes a portion of the originally denervated area. The reactive area of the band is therefore a reflection of the extent of the regenerated fibers. The portion of the band which has not been invaded by regenerative growth remains fully dark. The pale, or reactive area of the band increases daily at the expense of the dark, or unreactive area, and the process of nerve regeneration may be studied in the same animal, day by day, simply by repeating the above described procedure. The same phenomenon may be demonstrated in the reverse manner. Several animals in which nerve regeneration has begun at approximately the same time are made totally pale. Secondary incisions made anterior to the original transections are introduced at three-day intervals. The lengths of the dark streaks thus formed in the originally denervated band are measured distal to the initial incisions and the rate calculated. This average, and several other mean rates determined by still different methods, is a half-millimeter per day. Eventually nerves regenerate through the entire caudal band. This is complete within a month and at the end of this time all of the caudal band melanophores respond in a fashion similar to that of the unoperated portions of the tail (Figs. 1–6).

While nerve regeneration is progressing, however, the condition of the caudal band is such that part of it is normally innervated and therefore functional, and the remainder totally denervated (Fig. 7). Such preparations have been used in most of the experiments and will be referred to as " regenerating specimens." For convenience, that part of the band which has been innervated by regeneration will be called the " regenerated portion," and that part which has not yet received regenerated nerves, " the unregenerated portion."

B. Reactions of Melanophores in Blinded Animals

It was previously mentioned that blinded catfishes do not lose the capacity to undergo changes in shade. The mechanism by which such responses are brought about is not well known but is of considerable interest. Is the reactivity of the melanophores in blinded animals the result of the direct stimulation by light? This question is one of long standing, and no universal solution to it has been given. In the catfish, however, the following experiments show that the activity of melanophores in blinded animals is not a direct effect of light but is the result of a process which involves the transmission of impulses over the nervous system.

1. *Reactions of Innervated and Denervated Melanophores.*

a. Normal animals on white or black background and in darkness.— For purposes of comparison, a series of animals was tested after Parker's method. One-, two-, and three-ray transections were made in six white-adapted animals, and the animals subsequent to this treatment were maintained on a white background. The ultimate fading of the bands was completed after the following intervals:

Set 1 (1-ray transection)	2 days
Set 2 (2-ray transection)	5 days
Set 3 (3-ray transection)	10 days

Similar sets of animals maintained on a black background showed no changes after ten days. Another series placed in total darkness showed responses which were not uniform and perhaps deserve more detailed description. The catfish when kept in total darkness was reported by Parker (1934*a*) to be darker than intermediate controls. In another paper (Parker, Brown, and Odiorne, 1935) the animal was described as

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EXPLANATION OF PLATE I

All photographs are from life and of natural size. FIGS. 1–6 are photographs of the tail of the same catfish during regeneration of nerves to the caudal band.

FIG. 1. Tail of a white-adapted catfish one hour after a transverse incision was made across two rays.

FIG. 2. Same a week later.

FIG. 3. Same eighteen days after the incision was made.

FIG. 4. Same thirty-eight days after making the incision. Regeneration of nerves to the band had progressed eight millimeters as evidenced by the formation of a light band distal to the incision when the animal was placed on a white background for an hour. The dark region is the unregenerated portion of the band.

FIG. 5. Same forty-two days after the incision was made.

FIG. 6. Same forty-six days after the incision was made. Regeneration of nerves is almost complete.

FIG. 7. Tail of a light catfish in which regeneration of nerves to the caudal band was half completed. The dark portion is the unregenerated portion of the band.

FIG. 8. Tail of a blinded catfish kept in the dark room. Regeneration of nerves to the caudal band is three-quarters completed. The unregenerated portion of the band remains dark but becomes progressively pale as regeneration of nerves proceeds to the denervated melanophore.

FIG. 9. Tail of regenerated preparation treated with adrenalin (0.2 cc.). The photograph was taken three hours after the injection was made, during which time the animal was kept in black surroundings. The innervated areas darken, but the denervated (unregenerated portion) area remains pale for several hours longer.

FIGS. 10–12 are photographs of the tail of the same catfish.

FIG. 10. Tail of a regenerated preparation during electrical stimulation of the roof of the mouth for two minutes.

FIG. 11. Same five minutes after cessation of stimulation.

FIG. 12. Same ten minutes after cessation of stimulation. The unregenerated portion of the tail remains pale several hours longer.

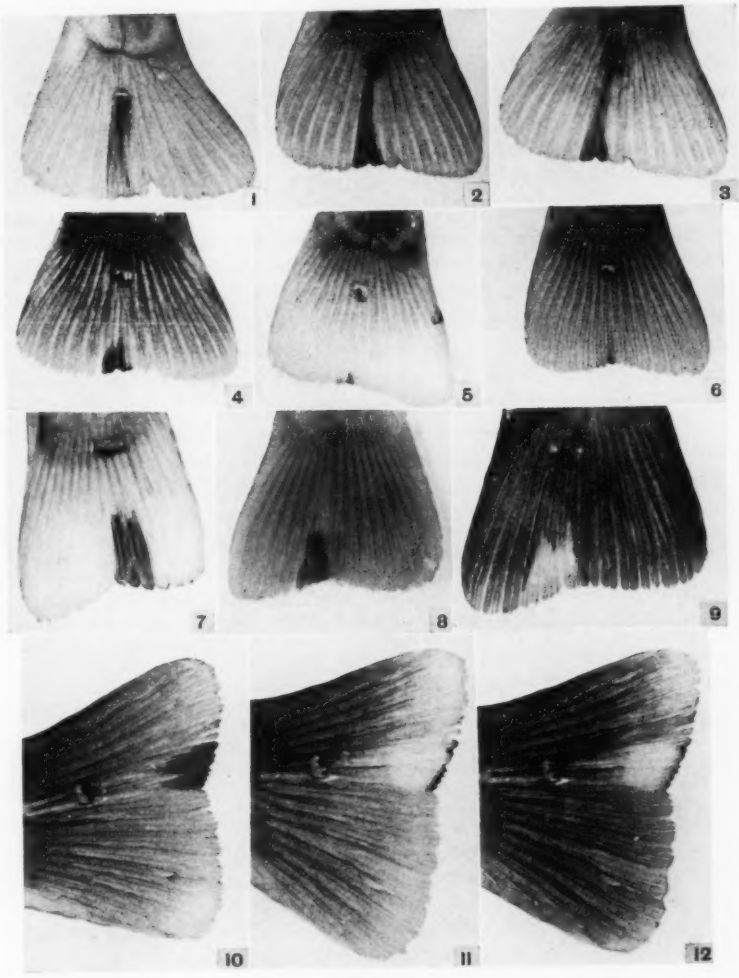

PLATE I

pale when kept under the same conditions. I have found no uniformity concerning this situation. Some animals kept in the darkroom were pale, though not as pale as blinded animals kept in the same room nor as pale as normal fishes kept in illuminated white vessels. The condition of the caudal bands in these animals was likewise variable. The bands were usually dark, but occasionally they were completely pale. Furthermore, bands which were pale one day were found to be fully dark on the following day, and vice versa. Observations were repeated many times during the course of a year in an attempt to obtain more uniform data, and while the tint of the animal as a whole was always close to an intermediate condition, that of the bands was extremely variable.

Since the results were not uniform, closer attention was paid to the condition of the animals before they were placed in total darkness. Hence animals in the two extreme states of background adaptation—very pale and extremely dark, with corresponding caudal bands—were placed in the darkroom for various periods of time. The following protocol is typical for a series of twelve animals.

PROTOCOL I

1933

December 25—10 A.M. Two fishes, one light-adapted for ten days with its induced caudal band faded, and the other very dark with its band equally as dark, were placed in the darkroom.

December 26—11 P.M. Both animals equally dark in tint, more to the dark side of the midpoint. Both caudal bands now darker than the rest of the animal.

December 28—7 P.M. Both animals light—more to the light side of the midpoint. Bands still deeply dark. (One of the animals became very excited during the observation, whereupon it paled markedly.)

December 29—11 A.M. Both fishes darker than previous observation, about equal in tint. Bands still darker than body tint. (One animal excited.)

December 30—1 P.M. Both equally dark. One band completely blanched in the animal which had been excited on the previous day; the other band as before.

January 1—10 A.M. One fish lighter than the other. Both bands darker than the general tint. (One fish excited again by the approach of the observer.)

January 3—11 A.M. One fish slightly lighter than the other, both about intermediate. One band dark, the other, in the fish which was previously excited, completely pale.

Fishes which were of intermediate tints and which exhibited fully dark bands when placed in darkness remained in much the same condition for eleven days except when disturbed by the approach of the observer.

These results indicate that regardless of the initial color (pale, intermediate, or black), the animals when placed in total darkness come to a state intermediate between the two extremes. This intermediate condition is not a constant one and the animals may be slightly paler or darker

than that which one may arbitrarily term an intermediate control. The fate of the bands is not consistent and depends in great measure upon the excitability of the animals. It is significant that a totally pale band will become fully dark when a pale animal is placed in total darkness. This takes place without any obvious stimulation and is interpreted as a capacity of denervated cells to expand in the absence of concentrating influences initiated by the eyes. Both observations—the behavior of denervated bands in excited animals and the darkening of pale bands in undisturbed specimens kept in the darkroom—will receive detailed treatment in the following pages.

b. Blinded animals on white or black background, and in darkness.—Blinded specimens, as Parker states, cannot respond to background influences. They therefore remained quite dark on either illuminated white or black backgrounds, and their caudal bands were equally dark. Blinded animals placed in darkness showed interesting responses. In all cases the fish as a whole was quite pale but the band was extremely dark. Such a condition persisted for twelve days at the end of which time regeneration of nerves into the denervated band commenced. As regeneration proceeded the dark band became progressively pale so that at the end of a month the entire tail was uniformly pale. These observations show that the pallor of blinded catfishes kept in darkness is a response mediated by the nervous system (Fig. 8).

Having shown that activity of melanophores in blinded animals in total darkness is dependent upon the nervous system in contrast to direct activity of the cells themselves, it became necessary to demonstrate that the effect of light was of a similar nature. When regenerating specimens of blinded catfishes kept in darkness were illuminated they became black within a minute or two. This reaction included the pale regenerated portion of the band. The unregenerated portion of the band, of course, suffered no change inasmuch as it was dark before the animal was illuminated. Such an experiment does not discriminate between the effects of light upon normal and denervated cells. If preparations could be made of blinded catfishes whose tails contained a band, the unregenerated portion of which was pale, the differential response of the innervated and denervated melanophores of the band would at once determine whether or not the effect of light is a nervous phenomenon. I have been unable to prepare blinded animals in this fashion, and consequently resorted to the use of freshly denervated bands. Several totally pale animals were quickly blinded and then returned to illuminated white vessels. Within thirty minutes, the animals became extremely dark but their denervated tail sectors remained totally pale. One hour later the bands became noticeably gray, and by the next day they were

as dark as the remainder of the integument. This condition persisted as long as they were illuminated, and the band showed no further changes. The response of pale blinded animals to light, like the reverse reaction, is carried out through nervous channels. Both sets of experiments confirm von Frisch's statement (1911), that the effect of light on the melanophores of most fishes is indirect.

2. *The Dispersed Condition of Denervated Melanophores.*—In the foregoing account a seemingly inscrutable situation has been disclosed. Animals when blinded and transferred to a darkroom attain a condition in which the denervated band is fully dark and the remainder of the animal pale. In the light of Parker's neurohumoral work, one would not expect such a condition to exist for more than two or more days; nevertheless, the band remains dark until nerve regeneration sets in some two weeks after the caudal bands were induced. The reason that denervated cells are fully and indefinitely expanded under such circumstances is not obvious unless it be that the natural tendency of a denervated chromatophore is expansion, and that this particular situation happens to be one which permits such an intrinsic property of a denervated pigment cell to assert itself. This explanation, admittedly, appears to constrain a compromise of an otherwise paradoxical situation; none the less, the following data fit such an explanation:

a. Normal animals.—In black-adapted animals the denervated band always appears as dark as the remainder of the animal. In any intermediate state the band is always darker than the general tint of the animal. In such animals of intermediate tints the greater expansion of the band melanophores cannot be due to the already-known dispersing or expanding agencies for if such were the case the band could not theoretically be darker than the remainder of the fish. Furthermore, the condition of the cells in the caudal sector cannot be an effect of the prolonged activity of severed nerves (Parker, 1934*b*), for the same condition prevails after these nerves must have degenerated completely. Finally, tail bands are darker than innervated regions of skin in animals maintained in the darkroom, except in special cases where it will be shown that the exception is the result of a different mechanism.

b. Blinded animals.—Here the evidence is more direct. Several animals kept on a white background for ten days were quickly blinded and placed immediately in total darkness. Their caudal sectors were, of course, fully pale in consequence of the prolonged white-background adaptation. Twenty-four hours later, these animals were found to be as pale as normal controls which had been white-adapted for ten days, yet their bands were extremely dark and remained in this state for two weeks until nerve regeneration proceeded to the band melanophores.

The full expansion of denervated melanophores occurs without any apparent stimulation, and seems to be an intrinsic property manifested only when such denervated cells are free of concentrating environment induced by the eyes.

3. *The Rôle of the Pituitary Gland.*—The experiments described in the preceding pages were performed on normal animals modified in particular aspects, and the results were presented as more or less conclusive of certain problems in the physiology of fish melanophores. In the final analysis, however, the results are not to be regarded as absolute because another melanin-dispersing agency, the pituitary gland, has not been taken into consideration. A series of experiments was therefore designed toward an analysis of the rôle of this gland in the chromatic responses of the catfish. Parker has attributed a minor rôle to the pituitary gland, inasmuch as neurohumoral responses occur in animals without pituitaries. It seemed desirable to repeat and extend Parker's observations on hypophysectomized animals, and to determine what influence the pituitary may have on the reactions recorded in the previous pages. About sixty hypophysectomized animals constituted the experimental material, and the results are in accord with what Parker has already established.

Twenty animals were maintained on white dishes until all were uniformly pale. Ten were hypophysectomized and returned to their white dishes. Three hours later denervated caudal bands (two-ray transections) were induced into all of the animals, after which they were left undisturbed. At the end of from five to six days the denervated bands faded in both the normal white-adapted and the hypophysectomized white-adapted animals. There was some individual variation, it is true, but there was no marked difference in the time of the end response of the denervated cells of both sets of animals.

Twenty animals were kept in black dishes for several days, and sixteen of these were hypophysectomized and returned to their original dishes. One hour later denervated bands equal to those of the white-adapted series were introduced, and the animals left undisturbed for six days. At the end of this time the four black-adapted normal animals, including their denervated bands, were entirely black. The hypophysectomized black-adapted animals were dark, though not black, and their bands were as dark or slightly darker than the remainder of the integument. Many animals exhibited a completely blanched tail sector, but this condition can be attributed to the mechanism described in the final section.

The environments of these two series of experimental animals were reversed, the white-adapted series was placed on black background, and

the black-adapted, on white. The white-adapted normal animals darkened within three hours; this response being shared by the denervated band. The white-adapted hypophysectomized animals darkened within three hours but the denervated band remained fully pale for several days, after which they assumed a moderate degree of melanophore expansion. The black-adapted normal specimens when transferred to the white background paled at the end of thirty-five hours; the band paled at the end of two and a half days. The black-adapted hypophysectomized animals lightened from one-half to eight hours, and their caudal bands bleached after a day. The pituitary gland is not indispensable for the color changes of this fish, yet it would seem from the above results that it acts as a "lubricant" for the machinery of melanin dispersion (especially for denervated cells) and as a counter-weight which the concentrating chromatophore nerves must overbalance to bring about melanophore contraction.

The previous work on blinded specimens showed that their reactions were under nervous control. It is conceivable, however, that light may stimulate photoreceptors of the skin which in turn would reflexly excite the pituitary to darken the animal and to maintain this dark coloration as long as the animal was illuminated. Such a process would necessarily preclude any discrimination in the responses of the innervated and denervated cells, for both types of melanophores should respond simultaneously to a blood-soluble substance which acts directly upon chromatophores. The matter was put to test by injecting into a totally pale specimen 0.2 cc. of commercial pituitrin:

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Injections were made at 11:25 P.M., and at 11:50 P.M. the fishes were decidedly darker. The bands darkened at the same pace as the rest of the animal. At 12:30 A.M. the fishes were uniformly very dark.

Both innervated and denervated melanophores expanded simultaneously and completely. Since illumination of blinded animals evokes a rapid expansion of normal melanophores, and leaves the denervated cells completely contracted for some time, it would seem that the pituitary could not be the agency responsible for the reactivity of the melanophores of blinded animals. This conclusion was confirmed by testing the reactions of blinded animals whose pituitary glands had been removed. As a matter of fact, von Frisch in his admirable work in melanophore physiology had performed this experiment twenty-five years ago, and his results were fully substantiated. Several blinded animals were maintained in total darkness and their reaction tested a half-dozen times to insure that they were typical specimens. They were then hypophysec-

tomized and returned to the darkroom. On the next day the tests were repeated. When illuminated they were found to be as pale as control specimens kept alongside in another dish, and within several minutes they turned quite dark. Again, this dark coloration was not as extreme as that which occurs when blinded (not hypophysectomized) animals are illuminated. Repeated tests showed that the blinded hypophysectomized fishes darkened to illumination, and paled after cessation of illumination.

We may now consider again the peculiar situation described previously, namely, the situation found in blinded animals kept in darkness where the innervated melanophores were contracted and the denervated melanophores were expanded. This same condition was found in similarly prepared but hypophysectomized specimens. Such specimens were uniformly pale except for the denervated band which appeared distinctly black, although not as black as that of blinded normal animals.[3] We are therefore left with no better explanation for this puzzling situation than that which has already been advanced, namely, the condition of a totally unstimulated denervated melanophore is one of pigment dispersion.

The receptor involved in the sensitivity of blinded catfishes to light was not investigated. Both von Frisch (1911) and Scharrer (1928) declared that the light receptor was situated in the vicinity of the diencephalon, but were unable to locate it precisely. Franz (1912) suggested that the so-called "neuropendymzellen im Thalamusependym" were light receptors in goldfishes. Scharrer (1932), impressed with the cytological secretory appearance of this region, wrote "im Reaktionsablauf sind wahrscheinlich nervöse und innersekretorische Vorgänge verknüpft." The peripheral route, however, does not seem to be hormonal, as the above experiments have indicated.

4. *Summary.*—All but one of the observations just described are easily understood. The nervous system directed by optic impulses is the controlling element in the chromatic responses of the catfish. This control Parker designates as neurohumoral since the eyes may eventually relegate the impulses they receive from the background throughout areas of denervated pigment cells. Blinded catfishes run the full gamut of pigmentary response from pigment concentration to pigment dispersion, and while this is a result of nervous stimulation, it does not include denervated cells. Neurohumoral responses are therefore normally lim-

[3] The reason that innervated and denervated regions of integument in hypophysectomized animals are not as black to the naked eye as those of normal animals was not determined. Although the condition of the bands and the normal areas of melanophores were described as dark or pale, microscopic examinations were made in most cases with the purpose of determining the relative degree of expansion or contraction of the dermal melanophores. Such factors as the behavior of the xanthophores and the epidermal melanophores which contribute to the external coloration of the animal await further investigation.

ited to animals with eyes. The pituitary is not an indispensable agency for melanin dispersion, yet it aids melanophore expansion especially for denervated cells, and by its presence hinders melanophore contraction resulting from efferent nerve impulses. Melanophores when totally unstimulated (denervated cells in blinded animals kept in darkness) remain indefinitely expanded. This condition seems to be due to an intrinsic property of a denervated cell. The sole observation which is not explicable by any of these mechanisms is the quick pallor of denervated bands and normal areas of the integument in emotionally disturbed animals as noted in Protocol I and elsewhere in the previous account. This matter may now be considered in detail.

C. Excitement Pallor

The notion that concentrating hormones similar to adrenalin (or probably adrenalin itself) may play a part in the color changes of fish is by no means novel. In 1911 von Frisch noticed that a dark denervated area in *Phoxinus* blanched quickly following excitation of the animal. Scharrer (1928) and Giersberg (1930) inclined to the view that such pallor must be due to endocrine factors acting directly on the chromatophores or on their nerve endings. Meyer (1931) was able to induce paling of dark specimens of *Gobius* and *Pleuronectes* by injecting serum from light specimens, and in the same year Smith (1931) established the necessity of the blood supply for the paling of dark ophthalmic areas (denervated) in *Phoxinus*. He concluded furthermore that the substance which effected a pallor in denervated regions is a hormone suspected to be adrenalin.

Observations of a somewhat similar nature were made by Bray (1918) in the catfish *Ameiurus*. Bray " raised sensitive individuals to a high pitch of nervous excitation," and found that the melanophores contracted to the maximum extent and remained in this state for a protracted period. This behavior Bray attributed to the eyes, for following etherization and removal of the eyes the melanophores became expanded, even if the fish was kept in the light. From such experimentation the conclusion was advanced that " there is a suggestion of the secretion of a hormone under certain conditions and of its influence on the melanophores."

I have frequently observed that catfishes paled when they were obviously disturbed by movements made in capturing them. Dark caudal bands which these animals possessed also bleached and remained in this condition several hours after the fish as a whole had darkened. Eventually, the band regained its former tint. The temporal sequence

of this process was as follows: Within two to five minutes after excitation of the animal, paling occurred throughout the fish except in the denervated band which retained its dark tint for from five to ten minutes longer. The band then faded rapidly and completely. Meanwhile, the body of the fish began to resume its initial dark color, but the band remained in a state of protracted pallor for from three to five hours. At the end of this time, the denervated caudal melanophores began to expand slowly (the animal being kept in black surroundings) and were fully expanded some three hours later.

Similar effects were evoked by electrical stimulation of the animal through the roof of the mouth, medulla or anterior end of the spinal cord. Electrical stimulation for two minutes resulted in a concentration of melanophore pigment in normal regions of the integument, and some five minutes later the denervated melanophores, dermal and epidermal, contracted in the manner already described (Figs. 10, 11, 12). Stimulation was carried out by the unipolar method, a lead plate on which the animals were placed being employed as a lead-off. Direct current (two volts, five amperes) was transformed by a Harvard inductorium. Animals placed in water between two stimulating electrodes also showed the same responses. These reactions were furthermore produced either by mechanical or electrical excitation of blinded specimens. The caudal band paled and darkened in the same period of time characteristic for normal animals, and thus contrary to Bray's contention that the eyes are necessary for pallor following excitation, both normal and blinded animals show the same reactions.

This rapid pallor of denervated sectors is apparently different from neurohumoral paling described by Parker. Since in all of the preparations the denervated areas were vascularized as well as the normal areas of the tail, and since the blood stream is the only quick avenue of approach to denervated cells, it was natural to suspect the blood stream as the agency responsible for the pallor following excitation. The temporal relations of the behavior of the innervated and denervated cells furthermore bring to mind the adrenalin vasodilator mechanism in mammals. A series of experiments was therefore directed toward a demonstration of the similarity between these two sets of observations. The experiments were divided into three groups: (1) a study of the reactions to adrenalin of innervated and denervated cells in fishes adapted to various backgrounds, (2) a comparison of the results with adrenalin with those produced by electrical stimulation, (3) a study of the blood of electrically stimulated animals.

1. *The Effect of Adrenalin.*—In the extensive number of papers which have dwelt upon the action of adrenalin on chromatophores, no

attention has been given to the "after-effects" of this substance on either the innervated or denervated cells. Thirty regenerating specimens were used in this series. The dosage of adrenalin chloride (Parke, Davis and Company) injected was 0.2 cc. of a solution diluted one part adrenalin to ten thousand parts water. The results are given in Table II.

TABLE II

Response of melanophores to intraperitoneal injections of adrenalin.

Condition of areas when injection was made		Time in minutes of response by concentration of pigment	Background to which fishes were returned	Time in hours of appearance of darkening "after-effects"	
Innervated	Denervated	Innervated and denervated		Innervated	Denervated
Black	Black	(10–21)	Black	3 hours	6 hours
Black	Black	(10–21)	Intermediate	3 hours	7 hours
Black	Black	(6–30)	White	Faint darkening*	No darkening
Light	Light	No perceptible change	White	No darkening	No darkening
Light	Light	No perceptible change	Black	4 hours	8½ hours

* This slight darkening was observed occasionally 2 hours after injection was made. It was only of short duration, however, for the animal soon paled in accordance with the white environment.

2. *The Effect of Electrical Stimulation.*—Fifteen regenerating specimens constituted the experimental material for this series. Results are arranged in tabular form in Table III.

TABLE III

Responses of melanophores to electrical stimulation of the roof of the mouth.

Condition of areas when stimulation was applied		Time of response by concentration of pigment		Background to which fishes were returned	Time of appearance of darkening "after-effects"	
Innervated	Denervated	Innervated	Denervated		Innervated	Denervated
Black	Black	1–2 minutes	4–11 minutes	Black	6 minutes	2–5 hours
Black	Black	1–2 minutes	4–11 minutes	Intermediate	5 minutes	2–5 hours
Light	Light	No change	No change	White	Darkening in 7 minutes*	No darkening
Light	Light	No change	No change	Black	Darkening in 8 minutes	4 hours
Black	Black	1–2 minutes	10 minutes	White	5 minutes. Slight darkening*	No darkening

* These darkenings were observed occasionally. They were only of short duration and the animals soon paled in accordance with the white background.

The more important results listed in the tables are as follows:

(1) Adrenalin—Denervated and innervated melanophores contract simultaneously following administration of adrenalin. Denervated cells once contracted by adrenalin never expand unless, following injection, the animals are returned to a black background. Innervated regions of integument sometimes darken slightly, despite the fact that the animal is maintained in white surroundings. There is thus a differential after-effect of adrenalin upon innervated and denervated melanophores.

(2) Electrical Stimulation—Denervated cells contract ten minutes after the innervated cells have responded. Such contracted denervated melanophores never expand unless, following stimulation, the animal is placed on a black background. The innervated melanophores always expand slightly following removal of stimulation, despite the fact that the animal is maintained in white surroundings. Thus after electrical stimulation there is a similar differential after-response of innervated and denervated melanophores. (Compare Fig. 9 with Fig. 12.)

The reactions of the innervated and denervated melanophores of catfishes to electrical stimulation, to adrenalin injections in proper doses, and to excitation produced mechanically are practically identical. Furthermore, they are not different from the responses of innervated and denervated blood vessels and other mammalian tissues (intestinal loops) subjected to treatment with adrenalin (Hartman and McPhedran, 1917*a*, *b*; Meltzer and Meltzer, 1903*a*, *b*, *c*). One is tempted to explain these results by assuming that excessive stimulation, electrical or otherwise, brings about a rapid nervous contraction of the innervated cells and a somewhat slower humoral contraction of the denervated cells. The humoral effect appears to be due to a substance which shares some physiological properties of adrenalin. Parallelism of tissue response, however, is no absolute criterion for the identification of an unknown material. The failure of known responses to occur in animals from which a suspected organ (the adrenals, in this case) is removed would be an important point in the elucidation of these psychic responses. Unfortunately, adrenalectomy in the catfish does not seem possible, and evidence must be obtained by indirect methods. The blood of excited animals was therefore investigated.

3. *Experiments with the Blood of Electrically Stimulated Fishes.*—Injections of 0.5 cc. of blood serum, or defibrinated blood, obtained from electrically stimulated fishes, were made under the skin near the dorsal fins of normal black-adapted animals. In twenty-two trials only three animals showed light areas at the point of injection. Although these injection experiments met with little success, the reverse procedure of excluding the blood supply to the tail proved more illuminating. Smith

(1931) had already shown that stimulation of *Phoxinus*, whose hearts had been previously removed, did not produce pallor of the denervated ophthalmic area. Similar results were obtained with the denervated caudal band in the catfish. While the catfish, whose heart had been previously removed, paled during electrical stimulation, the denervated area did not blanch in the time it would have blanched in a normal animal. The tail of a regenerating specimen was cut off distal to the posterior dorsal fin, thus removing the blood supply and all internal organs anterior to the region of the posterior dorsal fin. Stimulation at the base of the spinal column brought about a concentration of the innervated caudal cells in the usual time, but had no effect upon the expanded denervated cells. The rapid blanching of the denervating band in emotionally disturbed, or electrically stimulated animals seems to be due to a substance carried through the blood stream, and is therefore distinct from that which Parker has designated "neurohumoral contraction."

4. *The Effect of Electrical Stimulation in Blinded and Hypophysectomized Animals.*—Several tests, executed with no great detail, were performed on blinded regenerating specimens. Injections of adrenalin or electrical stimulation of blinded regenerating specimens produced responses entirely similar to those already described for normal fishes. The denervated bands always darkened from four to eight hours after the innervated melanophores had expanded following injection or stimulation.

Przibram (1932) published an account of experiments which attempt to harmonize conflicting effects of pituitary preparations on fish melanophores. Spaeth (1918), Matthews (1933), Hewer (1926*b*), Odiorne (1933), and Wyman (1924) reported melanophore contraction following pituitary treatment; Abolin (1925), Giersberg (1930), and Parker (1934*a*) found that pituitary injections induced melanophore expansion. Przibram brought these conflicting results together by showing that weak dosages of pituitary hormones induced melanophore expansion, but that stronger dosages evoked contraction. These findings are entertaining, and the possibility that the rapid pallor of the denervated bands may be due to an enormous secretory outburst of the pituitary gland following stimulation, electrical or mechanical, is plausible. This possibility is voided by the results obtained from the stimulation of isolated tails, and even more ruled out by the fact that the electrical stimulation or mechanical excitation of hypophysectomized animals brought about the same responses found in normal animals.

IV. Discussion

The experiments of Smith (1931), Parker (1934*a*), and those reported in the preceding pages show that several mechanisms may operate in the induction of chromatic responses in a particular animal. In the catfish the nervous system is the dominant mechanism, but humoral materials borne in the blood stream support and supplement it. The humoral and nervous action on melanophores differ in that hormonal action is slower and in that both innervated and denervated cells are primarily affected simultaneously. The humoral material which under special states induces pigment concentration has been shown to exhibit some properties characteristic of adrenalin. Such states have been called emotional, and the pallor resulting from this type of excitation has been termed excitement pallor (Redfield, 1918). Excitement pallor, however, has not only been completely denied by Zoond and Eyre (1934) for reptiles, but the senior author (Sand, 1935) has taken the diametrically opposite stand, namely, that excitement in reptiles results not in pallor but in darkening.

This aspect of melanophore physiology is therefore in a rather confused state, and the problem can scarcely be considered closed. The evidence that the adrenal glands are effective in the production of excitement pallor in reptiles comes chiefly from Redfield's work, which was accepted for some time by Hogben, but later denied by Hogben and Mirvish (1928*b*), Zoond and Eyre (1934), and Sand (1935). These investigators studied the African chameleon and were unable to confirm Redfield's findings in the horned toad. The toad and the Cape chameleon differ apparently in this respect. That two reptiles are not identical in their chromatic behavior is not really surprising, for when one considers the chromatic responses of the fishes one encounters an even greater variability. It is therefore difficult to maintain that the comparative chromatic apparatus is the same for members of a single class, and even more so for two classes of animals. Impressed with the universality of the anatomical basis and the bionomic aspect of color changes, Sand attempts a comprehensive equation for chromatic activity in fishes and reptiles and in so doing eliminates the possibility that the adrenal glands may be concerned with pigmentary activity.[4] The equation is not complete for all reptilian forms for Sand fails to include Noble and Bradley's (1933) finding that hypophysectomy results in a complete pallor of *Hemidactylus*. Furthermore, the existence of endocrine mechanisms for melanophore activity in some teleosts offers as

[4] Sand bases his views on Zoond and Eyre's disclosure of several inconsistencies in Redfield's data. It seems, however, that Zoond and Eyre have dealt with the exceptional cases in Redfield's data rather than with the more crucial experiments.

much of an obstacle for a universal equation of color changes in fishes as Redfield's data do for an all-nervous control in reptiles. The rapid blanching of denervated areas of skin in *Phoxinus* and *Ameiurus* seems to be explainable best on the assumption that a contracting substance reaches the experimental area through the blood stream. The assumption is strengthened by the fact that the denervated area does not blanch when the blood supply is cut off. Moreover, there are certain indications that the contracting substance is similar to adrenalin in pharmacodynamic effect. This is as far as the evidence goes for there is no proof that it is actually adrenalin, nor that, with the exception of Redfield's experiments with adrenalectomized toads, it is produced by the chromaffin tissue. These points must await further investigation, and the question of an adrenal influence in the color changes of fishes and reptiles is still open.

While the nature of the contracting blood-soluble material remains obscure, the phenomena with which this substance has been associated are of wider occurrence in fishes than in reptiles. Pouchet (1876) noted that excitement and fright resulted in the formation of dark spots on a pale turbot, an observation which Schaefer (1921) and Sumner (1911) also record for flatfishes. The stingfish darkened (von Frisch, 1912) but *Trigla*, the gurnard, and *Phoxinus*, the minnow, turned pale. My observations on *Holocentrus*, the squirrel fish of Bermuda, indicate that excitement results in the pallor of an otherwise red fish. These are several of the cases which may be cited, but it is apparent that there is no uniformity of psychic response. In those animals in which a general pallor occurred it must be remembered that no detailed discrimination, if any, was made between the reactions of the innervated and the denervated cells. Consequently, a comparison with the catfish cannot be made, for such general pallor following excitation of a fish may be due only to a nervous contraction of its melanophores. A denervated region of integument is necessary in order to determine whether excitation pallor occurs in denervated regions. If the denervated area shared the pallor following excitation, the agency must therefore be non-nervous and would be comparable to that which Redfield has advanced for the horned toad.

The occurrence of dark spots in frightened fishes, and a more general darkening of others are even further removed from the possibility of unifying psychic chromatic responses, yet one of my observations in the catfish may be of some interest. I have excited catfishes which possessed black caudal bands but which were otherwise totally pale. At the end of ten minutes of mechanical excitation, the caudal bands were completely blanched but the innervated regions of skin were darker than

the band and much darker than they had been before excitation. If the mechanism advanced for psychic response in the catfish is plausible, the darkening of normal areas of the integument may be a secondary effect brought about by the modification of the action of an adrenalin-like substance by the nervous system, and in the case of flatfishes, this secondary effect may be peculiarly restricted to small areas of the integument.

V. Conclusions

The factors which bring about changes in distribution of melanophore pigment are many. The eyes, as has been known for a long time, are the controlling elements for the adaptation of animals to black or white backgrounds. The control of the eyes is through the nervous system; yet Parker's work has shown that denervated cells may also respond to backgrounds, but this response occurs more slowly. The eyes, while essential for background adaptation, are not indispensable for the process of pigment migration in blinded but otherwise normal animals. Blinded animals may respond by full pigment concentration or dispersion, and while this response is nervous, it does not eventually include regions of denervated melanophores. Denervated melanophores of blinded fishes kept in darkness are expanded, despite the concentrated state of the innervated melanophores. Pale denervated bands become darker in blinded animals exposed to light, but there is no reason to suppose that this response is different from that which occurs when blinded specimens are kept in darkness. " Neurohumoral " responses therefore do not occur in blinded animals. The pituitary gland is not indispensable for expansion of innervated cells, but denervated cells are affected by pituitary insufficiency in that they do not readily attain a condition of full expansion in black-adapted hypophysectomized animals. Denervated melanophore areas bleach rapidly following excessive stimulation, electrical or mechanical. The agency responsible for such pallor may be a substance similar to adrenalin in pharmacodynamic effect.

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CUTANEOUS MELANOSIS IN LUNGFISHES (LEPIDOSIRENIDÆ)

G. M. SMITH AND C. W. COATES

(From the Department of Anatomy, Section of Neuro-Anatomy, Yale School of Medicine and Laboratory of the New York Aquarium)

During the past two years an opportunity has been afforded to observe two instances of a localized cutaneous melanosis occurring in lungfishes of the New York Aquarium. Both fishes were of the species *Lepidosiren paradoxa Fitzinger,* and had been caught in the Amazon River. On arrival in the New York Aquarium, the skin in each instance presented a normal dark slate color without any pigmented patches. The onset of pigmentation was regarded therefore as a spontaneous one.

Lungfish *A* (Plate I, Fig. 1) developed a slightly raised intensely black patch about an inch square in the right dorsal region above and encroaching upon the lateral line. It was situated approximately three inches from the head. Three months later, a piece of tissue a half-inch in length was removed for histological study, and at the same time a piece of the normal skin was excised for purposes of control. The wound in the normal skin regenerated without any unusual pigmentation, but the wound made in the melanotic region healed as skin with intensely black coloring. The fish remained alive for over a year following the biopsy without any further changes in the skin, the melanotic patch remaining the same size as originally seen.

Lungfish *B* showed a similar but slightly smaller black pigmented area in the right dorsal region above the lateral line about five inches from the head. This entire pigmented area was excised with a wide margin. Regeneration of the wound occurred in the course of the next four weeks, the regenerated skin appearing as normal looking skin without intensification of pigment.

Under aquarium conditions the skin of *Lepidosiren paradoxa* shows normally an epidermis composed of from four to six layers of epithelial cells, somewhat cuboidal near the free surface and polygonal or round near the basement membrane (Plate I, Fig. 3). Large mucous cells lie scattered throughout the epithelium, containing droplets of mucus which distend the cell and displace the nucleus toward the base. The epidermis rests upon a well developed basement membrane, below which lies a

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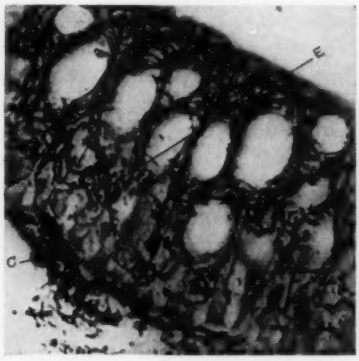

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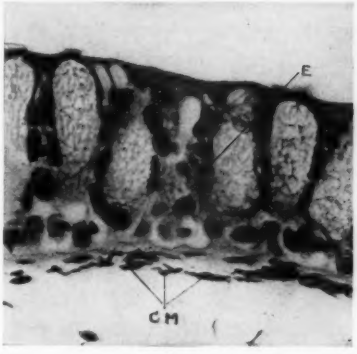

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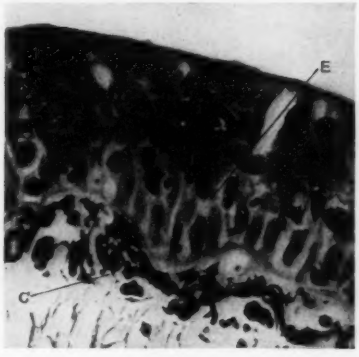

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PLATE I

EXPLANATION OF PLATE I

FIG. 1. *Lepidosiren paradoxa* (lungfish *A*) with pigmented patch (*X*) in the right dorsal region encroaching on the lateral line (*L*).

FIG. 2. Photomicrograph of melanotic tissue removed from lungfish *A*, showing a hyperplasia of melanophores infiltrating thickened epidermis (*E*). Numerous melanophores in the corium (*C*). Rogers' silver stain, counterstained faintly with erythrosin. × 250.

FIG. 3. Normal skin removed from lungfish *B*. *E*. epidermis; *CM*. corial melanophores. Hematoxylin and eosin. × 250.

FIG. 4. Tissue removed from melanotic patch of lungfish *B*. Mild increase of melanophores in corium *C* and a moderate infiltration of epidermis *E* by melanophores. Hematoxylin and eosin. × 250.

loosely arranged corium composed of interlacing fibrous tissue containing nerves and capillaries. Under normal conditions of pigmentation, the corium presents a moderate number of melanophores lying close under the basement membrane. Only occasionally are melanophores found among the epithelial cells of the epidermis.

The histological examination of the melanotic patches of skin removed from both the lungfishes, however, showed a greatly increased number of melanophores not alone in the corium but also infiltrating a somewhat thickened epidermis. These pathologic changes were noted particularly in lungfish *A* (Plate I, Fig. 2). A dense interlacing network of melanophores lies throughout all the layers of the epidermis. Intertwining dendritic processes of melanophores penetrate between epithelial cells and surround mucous cells, reaching actually to the free surface of the epithelium. The corium shows an increase over the normal numbers of melanophores, but adjacent muscle tissue was not invaded by pigment cells. The hyperplasia of melanophores was not associated with any leucocytic reaction. We found no evidence of parasites in the sections of diseased tissue examined. The melanophore infiltration of the epidermis of lungfish *B* was less extensive in character (Plate I, Fig. 4).

Discussion

Abnormal black pigmentation of the skin of fishes seems to depend on both genetic and post-embryonal factors. It may result from a more or less prolonged or permanent expansion of melanophores, or from an actual increase in the melanophores of the skin. Researches of Ballowitz (1893), von Frisch (1911) and other investigators have indicated that pathways exist between the brain through pigment motor nerve fibers to the sympathetic system, and from here by means of the peripheral nerves of the skin to the melanophores, affecting their contraction and expansion. Interruptions of these pathways by pathologic processes may result in an unusual black pigmentation of the skin produced by expansion of melanophores. Pathologic pigmentation of the skin caused by an increase in the number of melanophores occurs, for example, when certain parasitic larvæ gain access to the skin and become encysted. Mechanical trauma and X-raying have produced eruptions of corial melanophores and cutaneous pigmentation in the goldfish under experimental conditions (Smith, 1931, 1932). Yet in neither of the lungfishes exhibiting melanosis, kept for a long period of time in individual tanks, had there been any wounding of the skin.

Experimental studies covering a wide biological field attribute pigmentation to disturbances involving the endocrine system or enzyme

activity. Further investigations of melanosis of lungfishes may yield more definite information in these directions. It is unlikely that this, a distinct hyperplasia of pigment cells as well as of the epithelium, could be the result of humoral activity. In the present observation on two lungfishes, the stimulus to pigmentation remains undetected.

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A STUDY OF THE ENVIRONMENTAL CONDITIONS IN A BOG POND WITH SPECIAL REFERENCE TO THE DIURNAL VERTICAL DISTRIBUTION OF GONYOSTOMUM SEMEN

R. P. COWLES AND C. E. BRAMBEL

(From the Marine Biological Laboratory, Woods Hole, Mass., and the Johns Hopkins University, Baltimore, Md.)

Introduction

This is a study of a round, bog pond ("Cedar Pond") situated near the Marine Biological Laboratory in Woods Hole, Massachusetts. Well protected from winds by a wooded, sphagnum bog, the pond is ideal for a study of the vertical distribution of organisms. Higher chlorophyll-bearing plants were almost absent from the pond, during the summer of 1935, but large numbers of a chlorophyll-bearing flagellate, *Gonyostomum semen,* were present. The vertical distribution of this flagellate, the morphology of which has been described by Drouet and Cohen (1935), was studied together with such environmental factors as O_2, CO_2, pH, temperature, and illumination at the surface of the water.

No extensive discussion of previous work on vertical migration is undertaken in this paper, first, since the group of organisms studied is one not ordinarily investigated, and second, since it has been established so thoroughly that no one interpretation is possible for the various groups of animals and plants or for individual members of the same species (Parker, 1902; Esterly, 1907, 1917, 1919; Utermöhl, 1924; Whipple, 1927; Kikuchi, 1930; Worthington, 1931; Southern and Gardiner, 1932; Clarke, 1934; and Welch, 1935), and, third, since comprehensive reviews of the subject may be found in Rose, Russell, Whipple, Kikuchi, Clarke, and Welch.

Methods

The water samples, both for the study of organisms and for the chemical and physical tests, were collected with an Esmarch sampler which carries a 50-ml. sample bottle with a ground glass stopper. The temperature and pH readings of the water samples were made immediately after they were brought to the surface. Samples to be used for analysis for gas content were so collected that there were no air bubbles in the bottles, and they were kept packed in ice till the gas analyses were

made in the laboratory. The gas analyses were finished within two or three hours.

The degree of illumination was measured in foot-candles at the surface of the water, using a Weston photometer which was so constructed as to measure illumination from a few foot-candles to several thousand.

Since no extreme accuracy for temperature readings was thought necessary, they were made with an ordinary laboratory thermometer.

A Youden hydrogen ion apparatus with quinhydrone electrodes was used for determining the pH values of the water, corrections being made for temperature.

The dissolved oxygen and the total carbon dioxide (ΣCO_2) values, corrected for the usual interfering conditions, and for the H_2S in some samples, were determined with a Van Slyke manometric apparatus. In the earlier part of the work determinations were made in duplicate, but after it was found that values were reproducible, duplicates were determined only in those cases where the results were questionable. The free CO_2 and bicarbonate CO_2 were calculated using the proper equation (Clark, 1928). The carbonate content was considered negligible.

The organisms were counted under a compound microscope using a Sedgwick Rafter counting cell and a Whipple disc ocular. As a rule, total counts were made and the results were recorded in round numbers.

Physical and Chemical Conditions in the Bog Pond[1]

A study of the temperatures of the water from the surface to the bottom on August 20 and 21 (Table I) shows clearly, as would be expected, that the surface water increases in temperature from 5 A.M. to 1 P.M. but that by 5 P.M. it has begun a decrease which continues till the next morning. These changes are evidently brought about mainly by changes in air temperature. A similar but much slighter effect could be detected down to the 2.0-meter depth.

The wide range in water temperature, namely, from over 30 to 11° C. from surface to bottom (Table I), made it necessary to correct the pH values of the water for temperature at various depths. Our studies of the water of this pond show that it is acid in reaction. From the surface down to a depth of 1.0 meter the pH value is low (Table I) and much like that of the sphagnum bog water which surrounds the pond, i.e., it is distinctly acid with a pH value of 4.0 to 5.0. It is evident from the similarity in acidity of the sphagnum bog water (Drouet and Cohen, 1935) to that of the upper meter stratum of the pond, and from the increasing pH value of the water of the pond passing down-

[1] The authors are pleased to acknowledge having received advice from Dr. Robert Stiehler of the Wilmer Institute of the Johns Hopkins Hospital concerning certain chemical problems in this investigation.

ward below one meter, and from the existence of free communication between the sphagnum bog and the pond, that the upper meter of the pond water is largely of sphagnum bog origin. This conclusion supports the view of Jewell and Brown (1929).

Below the 1.0-meter depth the pH values for the water increase to over 6.0 usually and sometimes to almost 7.0 (Table I). Such an increase toward the neutral point at the bottom shows clearly that the greater acidity in the upper one meter of the bog pond does not have its origin from the bottom.

A diurnal change in the pH values is known to occur in certain bodies of water, especially fresh water, due to a decrease in the amount of CO_2 which is used up in the photosynthetic activity of the green organisms, although some of the decrease may be due to an increase in the temperature of the water with the consequent liberation of CO_2 into the air. Such a result has been reported by Bergman (1921), Cowles and Schwitalla (1923), Philip (1927), and Welch (1935). A glance at Table I shows this in the deeper levels of the pond, but above 2.0 meters such a condition is not so striking, probably because the acid water of the sphagnum bog, part of whose acidity is known to be due to some conditions other than the presence of free CO_2, keeps the pH value low throughout the whole twenty-four hours (Cowles and Brambel, 1934).

The determination of the CO_2 in the water of the bog pond, using the Van Slyke apparatus, gives us the total CO_2 (ΣCO_2) content, i.e., the sum of the free CO_2, bicarbonate CO_2, and carbonate CO_2. However, the low pH values of the upper layers, which consist mainly of sphagnum bog water which has seeped into the pond, show that the total CO_2 values of these layers are almost entirely free CO_2, since especially the carbonate and also the bicarbonate tend to be greatly reduced at such pH values. Below the 0.5-meter level bicarbonate becomes more important and this is indicated, in general, by the higher pH values (Table I). Water at these levels is largely of subterranean origin. Correlated with this difference in origin, the 0.5-meter water is characterized by only a trace of carbonate and only a small amount of bicarbonate with a ΣCO_2 of 16.2 p.p.m. The water of the 1.0-meter level has still only a trace of carbonate but a greatly increased amount of bicarbonate with a ΣCO_2 of 80.5 p.p.m. This sudden and extensive difference in total CO_2 content of the water passing through what may be called the transition layer is the most striking characteristic of the pond.

A diurnal variation in the ΣCO_2 is indicated fairly well in the surface water and it may be noted that the ΣCO_2 is very largely free CO_2,

owing to a low pH value resulting from some constituent other than CO_2 (Table I). The lowest ΣCO_2 value (almost entirely free CO_2) occurs in the late afternoon or early evening after the day's photosynthetic activity of *Gonyostomum* has about ended in the upper layers. On August 14 and 15 the same diurnal variation occurred. A similar condition at 0.5 meter existed on August 14 and 15 and very probably on August 20 and 21, but a very large irregularity in the 5 P.M. value on August 20 prevents us from drawing a positive conclusion concerning the latter observations.

The data show (Table I) that the free CO_2 content in general increases from the surface down to about 1.0 meter, as does also the total CO_2, and that the former constitutes a very large part of the latter. But beyond that depth the free CO_2 content decreases much although the total CO_2 content reaches its highest values at the bottom. Correlated with this relation, it is found that the bicarbonate CO_2 which is present in small amounts from the surface down to 1.0 meter, begins to increase in actual value, as well as to increase proportionally in value with the decrease in free CO_2. So in the upper layers the free CO_2 is far in excess of the bicarbonate CO_2 (Table I). At the bottom the bicarbonate CO_2 is far in excess of the free CO_2. But in passing toward the surface from the 1.5-meter layer to the 1.0-meter layer, where there is a distinct increase in acidity, which is indicated by the lower pH values, the shift from the condition in the lower layers to that in the upper ones occurs. Apparently in this region where the underlying real pond water, rich in bicarbonates, meets the more acid overlying water from the sphagnum bog, the acids react with the bicarbonates liberating CO_2 and the free CO_2 content of the water is thus increased and the bicarbonates decreased.

The dissolved free CO_2 of the surface water and in fact that of other levels is present in considerable amounts in the early morning (see also Welch, 1935), due to the respiration of organisms during the night, also to decomposition of organic matter in the water as well as at the bottom and likewise to the cessation largely, if not completely, of photosynthetic activity during the night. The decrease during the hours of the morning and usually during the early afternoon must be due to the photosynthetic activity of *Gonyostomum,* mainly, and to an increase in temperature which would tend to liberate some of the free CO_2 into the atmosphere. During the night photosynthesis ceases, or is greatly reduced and respiration increases, thus increasing the free CO_2 content of the water. The lowered temperature of the night helps to keep the CO_2 in solution (Table I).

The O_2 content of the water is about the same as that of small ponds

TABLE I

The diurnal and vertical distribution of Gonyostomum semen *in Cedar Bog Pond, Station D (see Fig. 2) on August 20 and 21, 1935, in relation to chemical and physical conditions.*

Time	Depth	Air temperature	Water temperature	pH	Total CO_2	Free CO_2	Bicarb. CO_2	Diss. O_2	Surface illumination	Gonyostomum
	meters	°C.	°C.		*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	*foot-candles*	*per 2 ml.*
5 A.M.	0.0	23	24	4.7	5.8	5.3	0.5	5.8	350	100
	0.5		23	4.7	16.2	14.7	1.5	1.7		1400
	1.0		20	4.9	80.5	69.5	11.0	1.2		1000
	1.5		17	5.2	83.0	63.0	20.0	0.6		200
	2.0		14	5.2	66.5	50.5	16.0	1.3		1100
	3.0		11	5.8	101.7	44.8	56.9	0.3		600
9 A.M.	0.0	28	27	4.7	4.6	4.2	0.4	6.2	3100	0
	0.5		24	4.9	12.8	11.1	1.7	2.4		1600
	1.0		15	5.1	79.5	63.5	16.0	1.3		400
	1.5		15	5.9	78.0	30.2	47.8	1.3		400
	2.0		14	6.0	78.0	26.0	52.0	0.6		600
	3.0		11	6.1	89.0	25.3	63.7	1.3		300
1 P.M.	0.0	30	30	4.6	7.5	6.9	0.6	4.7	3200	0
	0.5		25	4.6	11.6	10.7	0.9	2.7		900
	1.0		20	4.7	64.5	58.6	5.9	6.1		800
	1.5		18	5.2	85.0	64.5	20.5	0.3		400
	2.0		15	6.2	101.0	24.3	76.7	1.0		500
	3.0		11	6.4	70.5	11.6	58.9	1.3		500
5 P.M.	0.0	26	30	4.0	3.5	3.4	0.1	7.3	1000	0
	0.5		23	4.6	58.6?	54.3?	4.3?	3.3		100
	1.0		22	4.6	52.0	48.2	3.8	1.2		1000
	1.5		18	5.0	85.0	70.8	14.2	0.6		1200
	2.0		15	6.3	75.0	15.0	60.0	1.2		600
	3.0		11	6.6	105.0	11.7	93.3	1.3		900
9 P.M.	0.0	22	28	4.5	4.0	3.8	0.2	8.2	0	0
	0.5		25	4.7	8.1	7.3	0.8	2.8		0
	1.0		21	4.8	62.5	55.5	7.0	0.3		500
	1.5		17	5.0	88.0	73.3	14.7	0.3		800
	2.0		13	6.0	76.0	25.4	50.6	1.3		900
	3.0		11	6.0	96.0	32.1	63.9	1.3		900
1 A.M.	0.0	23	26	4.0	4.6	4.5	0.1	6.8	0	0
	0.5		24	4.0	13.3	12.9	0.4	1.3		100
	1.0		21	4.5	71.5	67.3	4.2	1.7		600
	1.5		17	5.5	79.0	49.0	30.0	1.0		900
	2.0		14	5.6	77.0	43.0	34.0	0.7		1000
	3.0		11	6.1	96.0	27.3	68.7	0.7		500
5 A.M.	0.0	22	25	4.3	5.8	5.6	0.2	7.2	150	200
	0.5		24	4.3	7.5	7.2	0.3	4.1		800
	1.0		20	4.5	79.5	74.6	4.9	1.0		1100
	1.5		17	5.0	84.5	70.4	14.1	1.3		700
	2.0		13	5.0	75.0	62.5	12.5	1.0		700
	3.0		11	6.1	102.0	29.0	73.0	1.0		300

in general. It is highest at the surface and decreases in most cases rather uniformly with the depth. The low O_2 content in the deeper layers is probably due, as suggested by Welch (1935) for other waters, to the presence there of considerable amounts of organic matter, to other gases rising in the form of bubbles from the bottom and very probably to an oxygen-depleted subterranean water which enters the pond at the

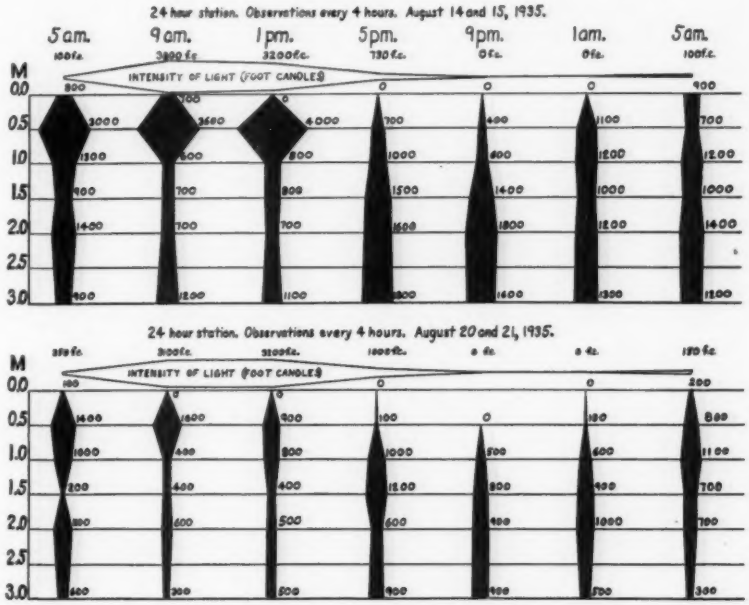

FIG. 1. This figure shows the vertical distribution of *Gonyostomum semen* from the surface to the bottom of Cedar Pond during two 24-hour periods. All the observations recorded in this figure are for the vertical distribution at Station *D*. In each observation the number of organisms in 2 ml. of water are shown. The degree of illumination in foot-candles at the surface of the water is shown for each observation.

bottom. Ordinarily there is a sharp decrease from the surface to the 0.5-meter level after which the decrease is much less, more gradual, and somewhat irregular, as can be seen from Table I.

A daily increase in dissolved oxygen has been found to occur on sunny days during the morning or morning and afternoon, especially at 0.5 meter (Table I). It is at the 0.5-meter depth that the maximum numbers of individuals of *Gonyostomum* (Fig. 1) were found during

the morning hours and it is reasonable to suppose that the increase in O_2 is due largely to the photosynthetic activity of that organism, although another supposedly chlorophyll-bearing flagellate found associated with *Gonyostomum* may possibly be another small source. It will be noted from Table I that the oxygen content decreases fairly regularly at the 0.5-meter level a short time after the *Gonyostomum* individuals begin their descent to the lower layers during the late afternoon (Fig. 1).

The average of the dissolved oxygen values on August 14 and 15, for Station *D*, from surface to bottom, at 1 P.M., as compared with the average at 1 A.M., are 2.7 p.p.m. and 2.1 p.p.m. Similar averages for August 20 and 21 are 2.7 p.p.m. and 2.0 p.p.m. These differences between the O_2 values at 1 P.M. and 1 A.M. are presumably due in a large part to a change in the photosynthetic activity of *Gonyostomum*.

The dissolved O_2 of the water of this bog pond and especially of the surface and 0.5-meter levels is considerably depleted early in the morning, e.g., 1 A.M. (Table I), due to the respiration of organisms and oxidation of organic matter in the water and at the bottom. During the morning or the morning and afternoon the dissolved O_2 in the water increases as the result, mostly, of the photosynthetic activity of *Gonyostomum*. The rise of temperature during this time favors the escape of O_2, but notwithstanding this, the increase in O_2 or "oxygen pulse," as it is sometimes called (Welch, 1935), is distinctly evident.

The data obtained with a Weston photometer for the illumination at the surface of the water are shown in Fig. 1 and Table I. The relation of these values to the distribution of *Gonyostomum* will be taken up in the next section.

Distribution of Gonyostomum

On an exceptionally bright morning it was found that at seven different stations distributed along a line extending across the bog pond the largest counts of swimming individuals of *Gonyostomum* were at a depth of 1.0 meter (Fig. 2). Judging from this widespread similar vertical distribution in the brownish colored water of this pond, it would seem then that under the physical and chemical conditions of that day and that time of day, a 1.0-meter depth from the surface offered the optimum conditions for these organisms. On other somewhat less bright mornings the maximum numbers were found at a depth of 0.5 meter (Fig. 1), while on a cloudy morning the maximum counts were at the surface. Muttkowski (1918) finds that the level for optimum photosynthetic activity for green plants in Lake Mendota, which is a clear, uncolored lake, is between 3 and 5 meters. Juday and

Schomer (1935) obtained similar results, by different methods, for other lakes.

The dependence of *Gonyostomum* on light as a stimulation for migration upward is strongly indicated, as one can see from the results shown in Fig. 1. At 5 o'clock in the morning, with only 200 to 300 foot-candles of illumination at the surface of the water, the *Gonyostomum* individuals were distinctly massed in the region of 0.5 meter below the surface and at 9:00 in the morning and at 1:00 in the afternoon, with an increasing illumination resulting in 3,000 foot-candles, 0.5 meter still seemed to offer the optimum conditions. But even at 1:00 P.M., on August 20, the counts indicated downward migration, since the number of organisms at 0.5 meter has decreased a little, while at

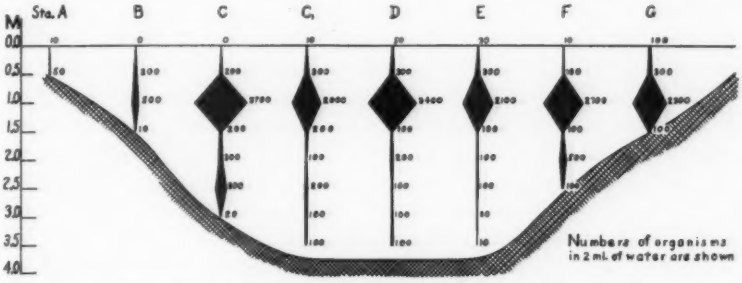

FIG. 2. This figure deals with the vertical distribution of *Gonyostomum semen* from the surface to the bottom of Cedar Pond from 9 A.M. to 10 A.M. on August 25, 1935. The vertical distribution is shown at Stations *A*, *B*, *C*, C_1, *D*, *E*, *F*, and *G* which lie along a straight line extending across the middle of the pond. The day was exceptionally bright and there were no clouds.

nearly all of the lower levels the numbers have increased. By 5 P.M., with a surface illumination of only a few foot-candles, the migration downward is indicated again by the higher counts at the lower levels and this condition continues at 9 P.M. when the photometer failed to show any appreciable illumination. It may be seen that in the case of both of the twenty-four-hour periods during which studies were made, at 5 P.M. and 9 P.M., the bottom (3.0 meter) counts were the largest when compared with those for any other time during the whole twenty-four hours at that depth. This increase in the lower layers with a corresponding decrease in the upper layers is almost positive proof that there has been an extensive migration or a quiescent sinking of the individuals of *Gonyostomum* toward the bottom. In this paper the bottom of the pond is spoken of as being at 3.0 meters—the lowest depth at which satisfactory

samples could be taken in routine work. Between this depth and 3.5 meters, where there is a somewhat solid bed of decaying vegetation, there is a mush of dark brown mud and frequently of vegetation where, ordinarily, it is a very laborious process and one of questionable accuracy to make counts of organisms.

It is quite surprising to find that by 1 A.M. both on August 15 and August 21, individuals of *Gonyostomum* began to increase again in the upper layers and decrease at the lower levels (Fig. 1), indicating an upward migration. This migration, at 1 A.M., was more marked on August 15 than on August 21 and correlated with this difference, our records show that there was bright moonlight on the night of August 14–15, while on the night of August 20–21 there was much fog although at times the moon was to be seen. While our observations concerning the effect of moonlight on the behavior of *Gonyostomum* are meager, they suggest that these organisms react to the moonlight and migrate toward the surface, when there is moonlight during the early morning hours. Such a suggestion as to the cause of this behavior is supported by the observations just mentioned, concerning the illumination due to moonlight on two different nights, as compared with the numbers of *Gonyostomum* individuals to be found in the upper levels of this pond.

In the opinion of the authors, the observations made and our knowledge of the structure and behavior of *Gonyostomum* indicate that light is an important factor in the upward migration of that organism. It would seem that having spent several hours in darkness and, as a result, having probably become exceptionally sensitive to light, it tends to migrate upward till it reaches the proper combination of illumination, free CO_2 and O_2 content, and temperature, which are necessary for the required photosynthetic activity.

The downward movement in the early afternoon, sometimes as early as 1 P.M. when the light is still bright, is rather surprising. One might suspect that having carried on the process of photosynthesis long enough to satisfy their carbohydrate needs, the *Gonyostomum* individuals swam or sank quiescently toward the bottom. Drouet and Cohen's (1935) observation and our own have shown that they do sink to the bottom of jars in the laboratory.

We are dealing with a periodic phenomenon here—a migration to the upper levels during the morning and a movement toward the bottom in the afternoon and evening. The theory that this may be regulated by some internal condition of the organism, by some periodic physiological change which regulates the periodicity in behavior, must not be disregarded. There is considerable evidence to show, in the case of cer-

tain snails and green unicellular forms, that periodic changes in behavior which are correlated with definite periodic changes in the environment occur even when the organisms are no longer subjected to these changes in the environment. Mast (1920), studying a euglenoid form, *Lepocinclis texta,* found a periodic reaction toward light and it is very probable that the periodic movement of *Gonyostomum* away from the light, at least, involves a periodic physiological change in the organism—possibly a cessation of photosynthetic activity when sufficient sugar has been formed, such as we have mentioned above, or a reproductive change or both. In fact, it is known that *Gonyostomum* sinks to the bottom and undergoes longitudinal division.

It has been shown by several investigators (see Mast, 1911) that changes in temperature may bring about reversal in the direction of the response to light, but the diurnal changes in the bog pond under consideration do not seem to be important since the decrease in the water temperature as day changes to night is not very great at any one level except at the surface, where, as our studies show, there are ordinarily few individuals of *Gonyostomum.* However, the range of temperatures from surface to bottom is large (Table I) and it is evident that *Gonyostomum* is subjected to rather large changes in water temperature during its movements, so that since a higher temperature often causes green, flagellate forms to react positively to light, the temperature may be a factor which helps to determine the upward migration.

Since we are dealing with an organism which carries on photosynthesis, water temperature must necessarily be an important factor, for it is known that photosynthesis takes place within certain temperature limits. It is surprising to find that during the daytime the congregation of *Gonyostomum* individuals in large numbers along a vertical gradient almost invariably takes place in regions where the temperature is about 24° C. It may be assumed, although not proved, that during the daytime where this congregation in large numbers occurs, photosynthesis is at its height. Numerous observations, not recorded in the text, in which the organisms under discussion were found congregated together during the daytime in large numbers in water of about 24° C., point strongly to temperature as being a factor in determining the vertical distribution, but it does not rule out other factors such as the sunlight, reproductive periodicity, CO_2 content and O_2 content of the water, etc., because we find that during part of the twenty-four hours, namely, the hours of darkness, when photosynthesis has stopped or has been reduced to a minimum, the congregation of *Gonyostomum* individuals in largest numbers does not take place in water of a temperature of 24° C.

There is no intention of offering these results as proof that *Gony-*

ostomum reaches its "optimum" photosynthetic activity at about 24° C., since we have no experimental work to support it and since it is well known that such a so-called "optimum" may vary with the amount, intensity, or quality of other factors concerned in photosynthesis. However, our water temperature records must be taken into consideration in any attempted explanation of the vertical distribution of this organism.

Without having determined experimentally the amount of CO_2 used by *Gonyostomum* in photosynthesis, but judging from the abundance with which this organism occurs at most levels in the pond, it would seem that there is a plentiful supply of CO_2 for their needs from the surface to the bottom. It will be remembered that practically all of this is in the form of free CO_2 and bicarbonate CO_2, both of which can be used by chlorophyll-bearing organisms. The congregation of *Gonyostomum* individuals in rather large numbers at lower levels during the night, when the free CO_2 content of the water may reach 75 p.p.m. or more, indicates that such amounts of free CO_2 are not particularly detrimental to these green flagellates. In fact the decreasing amounts of free CO_2 during the daylight morning hours, from 5 A.M. to 9 A.M., at all levels and the increasing amounts of free CO_2, with one exception, during the dark morning hours, from 1 A.M. to 5 A.M., indicate that photosynthesis takes place at all depths during the daytime. These conditions may be seen in Table I where the total CO_2, free CO_2, and bicarbonate CO_2 are given in parts per million for August 20 and 21, at various depths and times of day.

It must be pointed out that our results are not offered as conclusive proof that photosynthesis takes place at all depths during the daytime. In fact, we know that at times bubbles of gas rise to the surface of the pond which should tend to cause irregularities. However, we think that our results indicate that photosynthesis may occur at all levels.

Since the knowledge of how free CO_2 and bicarbonate CO_2 are used by aquatic, chlorophyll-bearing organisms is quite uncertain and since our studies of the relation between the CO_2 content of the water and photosynthesis have not been experimental in nature, we shall have nothing more to say concerning the relation between the CO_2 content of the water and the vertical distribution of *Gonyostomum* except in so far as to point out that the amount of free CO_2 is one of the factors governing photosynthetic activity.

We can say but little concerning the effect of the oxygen content of the water on the movements of *Gonyostomum*. However, if it can be demonstrated that this organism behaves like the chlorophyll-bearing *Paramecium bursaria* which, according to Engelman (1882) moves to-

ward the light, when there is little oxygen in the water in which it is swimming, so that under the increased intensity the organism will produce more oxygen for respiration as a result of increased photosynthesis, then the small amounts of dissolved oxygen in the lower layers may possibly be considered as a factor in determining the upward migration of *Gonyostomum* individuals during the early morning hours.

Our observations furnish no evidence to support the idea that the organism under consideration migrates vertically due to a search for organic food.

Summary

1. The vertical distribution of the green, euglenoid-like organism, *Gonyostomum semen,* in a bog pond, has been studied at intervals of four hours, throughout twenty-four hours, with reference to air temperature, water temperature, illumination at the surface of the pond, total-, free- and bicarbonate CO_2 in the water, O_2 in the water and the pH value of the water.

2. The practically undisturbed condition of the water in the pond made it possible to demonstrate an oxygen and a carbon dioxide "pulse." These are mainly the result of the photosynthetic activities of *Gonyostomum.*

3. The acidity of this bog pond is partly due to the acid water which enters from the sphagnum bog.

4. A striking chemical characteristic of the pond is the sudden increase in the free CO_2 content of the water between 0.5 and 1.0 meter.

5. Many individuals of the *Gonyostomum* population of this pond move upward during the early morning hours and congregate in maximum numbers somewhere between one meter and the surface. The level at which this occurs seems to be dependent on the proper combination of light, water temperature, and CO_2 content of the water. During the late afternoon and night many individuals move downward so that the maximum counts are found in the lower layers. The upward movement of individuals of *Gonyostomum* from the lower levels is probably due to an increase in the light at the source combined with an increasing gradient of light, temperature, and CO_2 from the bottom to the surface, acting on *Gonyostomum* individuals which have been living in darkness.

The downward movement is probably due to some physiological condition resulting from a sufficient amount of photosynthetic activity or a reproductive tendency or both which is followed by a quiescent sinking.

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PROTEIN LIPID BINDING IN PROTOPLASM

L. V. HEILBRUNN

UNIVERSITY OF PENNSYLVANIA

Eleven years ago, Leathes and Raper (1925) wrote: "Clearer ideas as to the physical relation between insoluble fat and the aqueous protoplasmic systems of the living organism, and of the part played by cholesterol in this relationship, are indispensably necessary before we can answer some of the commonest questions in physiological inquiry." The sentence is as true today as when it was written. Although it is now generally realized that protoplasm is more than a solution of proteins, and that non-aqueous lipids are essential to its life, biologists and physiologists have scarcely given thought to the problem of how the lipids are related to the rest of the protoplasm. Most students of the cell are scarcely aware of the existence of the problem. And yet the pathologists have known for many years that the fats and fat-like substances of protoplasm are so bound or united to proteins as to be for the most part non-recognizable in the living or stained cell. It is only when degeneration occurs that the lipids are freed from their union with protein. In such degenerated cells, fats previously concealed may occupy a large part of the cell volume. Obviously, such a freeing of lipids results in an impairment or a loss of protoplasmic activity, and this bears witness to the importance of the lipids for the vital machinery.

The manner in which lipids are bound to proteins in living cells is thus a question which merits investigation, and as will be shown later, it is a question which can be studied experimentally. Unfortunately, however, one difficulty in the way of progress is the lack of definite information as to the manner in which various lipids may unite with proteins in vitro. Thus Handovsky (1933) states: "Die Verbindungen die einzelne Eiweisskörper mit anderen Eiweisskörper oder mit anderen lyophilen Kolloiden (z. B. Lipoiden) eingehen, sind biologisch sehr wichtig, aber wenig exakt untersucht."

The experiments to be reported in this paper were begun as the result of a chance observation. Sea urchin (*Arbacia*) eggs which had been exposed for 4 hours to a solution of oxalated sea water made by adding 1 part of m/4 ammonium oxalate to 2 parts of sea water were centrifuged in an Emerson electric centrifuge (force about 7,000 times gravity). It was then found that the gray cap of these treated eggs was

very much larger than that of normal eggs. As is well known, the gray cap of centrifuged *Arbacia* eggs consists of lipid substances. There was thus an apparent increase of the lipid portion of the protoplasm. In the original experiment fertilized eggs were placed in the solution, but later observations showed similar, though not as pronounced effects with unfertilized eggs. With the unfertilized eggs, somewhat longer exposures are necessary.

It was first thought that the action of the oxalated sea water was due to the oxalate ion. However, experiments with potassium oxalate gave no support to this view, and it was realized that the effect produced by the ammonium oxalate was due to the ammonium ion. Indeed, various types of ammonium salts all give essentially the same results. In addition to the oxalate, the acetate, chloride, and sulfate were successfully used.

FIG. 1

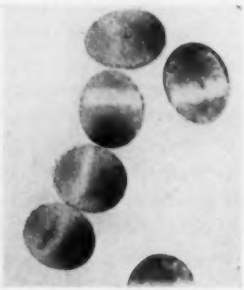

FIG. 2

FIG. 3

The details of the experiment are very simple. Eggs are placed in mixtures of sea water and isotonic solutions of an ammonium salt, and are centrifuged after the lapse of a suitable time interval. Solutions of ammonium salts alone can not be used, for they cause a coagulation of the protoplasm, and the eggs then resist stratification on centrifugal treatment. In most of the experiments, one part of an isotonic solution of ammonium chloride was added to 3 or 4 parts of sea water, and eggs were then immersed in this mixture. If unfertilized eggs are used, in 4 or 5 hours, centrifugal treatment shows an increase in the volume of the gray cap. A very marked increase appears after 8 or 10 hours. A typical result is shown in Figs. 1–3. These show eggs which were centrifuged for 3 minutes in an Emerson electric centrifuge at about 7,000 times gravity. The control eggs shown in Fig. 1 were allowed to lie in sea water for 8 hours before being centrifuged; they show a typical

small gray cap. Figs. 2 and 3 show the appearance of eggs centrifuged after exposure to 70 parts sea water plus 30 parts 0.53 m NH_4Cl for 8 hours. The centrifugal treatment of the experimental eggs was the same as that of the control, but the eggs exposed to the ammonium salt show a greatly enlarged gray cap. Indeed the gray cap is so large that the hyaline zone is reduced to a narrow streak.

It might be thought that in these eggs with enlarged gray cap, the increase in volume is due to a greater scattering of the materials of the cap. However, when the eggs are centrifuged for short or long periods, the volume of the gray cap remains essentially the same. Such centrifugal treatment can not be continued indefinitely, for the eggs eventually break into two; but with the most vigorous centrifugal treatment possible, the gray cap of the eggs exposed to ammonium salts is always much larger than that of normal eggs.

The behavior of *Arbacia* eggs toward solutions of ammonium salts is not peculiar to this type of material. When eggs of the clam *Cumingia* are exposed to mixtures of sea water and isotonic ammonium chloride or oxalate, there is in this case also an increase in the width of the fatty zone when the eggs are centrifuged. This increase is not as striking as in the case of *Arbacia*. In *Cumingia* eggs, the normal gray cap or zone of fatty materials appears rather dark under the microscope. What appears to be the added accumulation after treatment with ammonium salts is somewhat lighter or more transparent. A similar distinction can sometimes be observed in *Arbacia* eggs.

Ammonium salts may also cause an increase in the visible fat of *Amœba proteus*. In experiments performed in May, 1935, amœbæ were kept in dilute solutions of ammonium chloride for 3 or 4 hours and were then centrifuged vigorously (5 minutes at 7,000 times gravity). The solutions used were m/60 or m/120 NH_4Cl, at a pH of approximately 6.0. In one case, 1 part of m/60 NH_4Cl was added to 2 parts of m/40 NaCl (pH 6.5). Numerous experiments were tried. In every case, care was taken to select cultures which normally had no free fat or lipid in them, as occasionally cultures are found which contain free fat even without treatment. Normally, when an *Amœba proteus* is centrifuged vigorously, the crystals of the protoplasm accumulate in the heavy or centrifugal half of the cell and the rest of the cell is clear and transparent (see Heilbrunn and Daugherty, 1932). However, after treatment with ammonium salts, the centrifuged amœbæ had approximately a fourth or more of the cell filled with particles lighter than the rest of the fluid protoplasm. These light particles stained brilliantly with Sudan III and were thus clearly lipoid.

Amœbæ constitute a much more variable material than *Arbacia* eggs.

It has already been noted that occasional cultures show free fat even without treatment with ammonium salts. On the other hand, in experiments done in March, 1936, no free fat could be demonstrated even after treatment with ammonium salts. However, in June, 1936, experiments with ammonium chloride were successful in 80 per cent of the amœbæ tested. Perhaps there is a seasonal variation. Whether this is true or not, one can distinguish three types of amœbæ. In the first type, free fat is present normally; in the second, free fat appears only after treatment with ammonium salts; and finally there are amœbæ in which there is no free fat even after exposure to ammonium salts.

Similar variations appear to occur in frog muscle. Students in my laboratory have studied the effect of ammonium salts on the detectable fat in frog muscle fibers (*Rana pipiens*). Mr. H. Blumenthal exposed gastrocnemius muscle to isotonic solutions of ammonium salts mixed with Ringer's solution, and he found on fixing in osmic acid solutions that there was a great increase in the free fat. Similar results were also obtained with heart muscle of the frog. Mr. Newman has repeated Blumenthal's experiments using single muscle fiber preparations from the frog sartorius, and staining with Sudan III. In the winter, the normal muscle fibers are without free fat, but show a large amount after exposure to ammonium salts. In the spring, the normal muscle fibers have free fat even before treatment. In the late summer, it is possible that free fat may be absent even after ammonium treatment. This would accord with the observation of Bell (1911) that during the summer months it is not ordinarily possible to demonstrate fat-staining granules in *Rana pipiens* muscle, although such granules are easy to observe during the spring and fall.

It is a remarkable fact that in various types of protoplasm, in sea urchin eggs, in clam eggs, in amœba, and in frog muscle, treatment with ammonium salts results in an increase in the free fat or lipid. In the sea urchin egg, this increase in fatty materials is indicated by a very marked increase in the volume of granules lighter than the rest of the protoplasm. That these lighter granules are fatty seems reasonably certain. They could scarcely be protein, for proteins constitute the heaviest rather than the lightest constituents of the protoplasm. An attempt was made to stain the lighter granules of the sea urchin egg with Sudan III. This did not prove very satisfactory, perhaps because of the small size of the granules. In amœba, on the other hand, the light granules set free following treatment with ammonium salts stained very readily with Sudan III. So, too, in the muscle experiments, fat could be demonstrated both by osmic acid and by Sudan III.

It might be argued that in the experiments with *Arbacia* eggs, the

increase in volume of the oil cap was due to an increased affinity of the oil for water. However, if *Arbacia* eggs are broken into two fragments by very vigorous centrifuging, and the heavier fragments which are free from fat or oil are then treated with ammonium salts, there is an accumulation of lighter particles at the centripetal pole of the heavy fragments when these are centrifuged. This indicates that the lipid material which appears following ammonium salt treatment can originate from protoplasm freed from its original fatty substances. Moreover, it may be noted again that in amœba, cells totally without free fat may be made to show a considerable quantity after treatment with ammonium salts.

How can one account for the increase in lipid materials? Although change from protein to fat is possible, it can readily be shown that no such transformation is responsible for the results outlined above. By gathering large masses of eggs, it was possible to make quantitative determinations of the total lipid content of normal eggs as compared with the lipid content of eggs treated with ammonium salts. In order to insure an effect of the ammonium salts, the eggs must be placed in large flat dishes so as to avoid crowding during the experiment. A concentrated egg suspension obtained from a hundred or more sea urchins was divided into two equal portions. One portion was allowed to remain in sea water. To the other portion, 0.53 m NH_4Cl was added in the proportion of 3 parts of the solution to 7 parts of sea water. After 8 hours, the eggs from each portion were collected and analyzed for total lipid. In one experiment, extraction was performed with a modified Soxhlet apparatus, in a second the wet material was extracted with alcohol and ether. Both tests showed almost identical quantities of total lipid in eggs treated with ammonium salt and controls. These tests were performed with the assistance of Mr. Samuel Koppelman.

As a result of the chemical analyses, it may be concluded that the action of ammonium salts is to free bound lipid from union with protein. That living cells normally contain a relatively large amount of fat or lipid in the bound state is abundantly clear from the literature on fatty degeneration. Cells may appear to be practically free from fatty substances, whereas upon degeneration they become filled with numerous fatty droplets. And yet, often in these cells which have undergone fatty degeneration, no increase in total lipid can be demonstrated by chemical methods. Thus, obviously, bound fats or lipids occur in protoplasm. In blood, also, the lipids are to a large extent bound to the proteins.

The mechanism of action of the ammonium salts is to some extent clear. Jacobs (1922) has shown that when cells are exposed to a medium containing ammonium salts, the pH of the protoplasm rises. This is due to a hydrolysis of ammonium salt to ammonium hydroxide and

acid. The ammonia or ammonium hydroxide is then able to penetrate the cell, whereas the inorganic acid remains outside.

That the effect of the ammonium salts is in reality due to an alkalinization of the protoplasm is supported by two sets of observations. In the first place, ammonium hydroxide also causes an increase in free or visible lipid. Thus sea urchin eggs were exposed for several hours to solutions with enough ammonium hydroxide added to bring the pH to 8.9 in one case, and to 9.2 in another. To avoid excessive precipitation of magnesium hydroxide, the ammonium hydroxide was not added to sea water, but to a sodium chloride-calcium chloride mixture (125 cc. 0.53 m NaCl + 1.5 cc. 0.3 m $CaCl_2$). When the eggs were centrifuged after exposure to these solutions, there was a marked increase in the volume of the oil cap, and the appearance of the eggs was like that shown in Figs. 2 and 3.

In the second place, it was discovered that an excessive amount of carbon dioxide in the medium surrounding the cells prevented an increase in free fat or lipid. Thus when egg cells were crowded, even though they were in contact with the proper strength of ammonium chloride, no increase in visible fat occurred. Also when carbon dioxide was bubbled through the medium, the action of the ammonium chloride was inhibited. Carbon dioxide readily penetrates cells and it doubtless tended to prevent the alkalinization of the protoplasm which might otherwise have been caused by the ammonia diffusing into the cells from the ammonium chloride solution.

It is quite conceivable that alkalinization in itself might cause a liberation of lipid from protein-lipid combination. Thus Theorell (1930), who has done some of the most interesting work in the little-studied field of protein-lipid binding, states:

"In der Literatur findet man mehrere experimentell mehr oder weniger wohlbegründete Angaben, das sowohl Cholesterin als auch Phosphatide an die Globuline, besonders an das Euglobulin, gebunden seien. Gäbe es eine solche Verbindung, wäre es ja nicht unwahrscheinlich, dass sie bei dem isoelektrischen Punkte der Globuline dissoziiert wäre, wo ja ihr Minimum an elektrischer Ladung und Hydratation zu finden ist."

Theorell himself studied the binding of globulin and cholesterol and found that although the cholesterol was bound at either side of the isoelectric point, at the isoelectric point itself there was no binding. However, in the case of albumin the situation is not quite so simple, and a recent paper of von Pryzlecki, Hofer, and Frajberger-Grynberg (1935) indicates the complexities of protein-lipid binding.

In protoplasm, it is quite possible that increase in pH does bring a shift toward the isoelectric point, of at least certain of the protein con-

stituents. In this connection, it is interesting to remember that there is evidence that protoplasmic particles migrate to the cathode when an electric current passes through a cell (see Heilbrunn, 1928). This would indicate that there are proteins present which are on the acid side of the isoelectric point.

Summary

1. When various types of protoplasm are treated with dilute solutions of ammonium salts, there is an increase in the free fat or lipid.

2. This effect is due to an alkalinization of the protoplasm.

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FREE CALCIUM IN THE ACTION OF STIMULATING AGENTS ON ELODEA CELLS

DANIEL MAZIA AND JEAN M. CLARK

(From the Zoölogical Laboratory, University of Pennsylvania)

Introduction

A number of investigations have been made in this laboratory on the colloid-chemical changes in the protoplasm that are involved in cell stimulation. The results conform to the hypothesis, discussed most fully by Heilbrunn and Daugherty (1933), that a primary effect of stimulation is the release of Ca ion in the cell. Heilbrunn, Mazia, and Steinbach (1934) (fuller accounts to be published soon) reported that direct analysis after ultrafiltration showed that in the fertilization of sea-urchin eggs and in the injury of muscle, the free Ca content of the cells indeed increases. On the other hand, changes which are interpreted as evidence of Ca release are prevented by oxalates (Heilbrunn and Young, 1930; Heilbrunn and Daugherty, 1933).

At the present stage of the problem, a qualitative, intracellular method would be even more valuable than our quantitative ultrafiltration method for a general testing of the hypothesis of Ca release. The chemical accuracy of the latter method is offset by the fact that masses of cells must be used, and must be subjected to violent means of killing. An intracellular method in which it is not necessary to add reagents is ideal. Just such a method is provided in the present case by plant cells which contain high concentrations of soluble oxalates in their sap. An increase in the free calcium concentration of the cell is immediately indicated by the formation of calcium oxalate crystals.

Although there is a large literature on the natural occurrence of calcium oxalate, dating back to Malpighi (1675) (*cf.* Patschovsky, 1920), there are very few papers on its experimental production in cells in which it does not normally occur. Osterhout (1910) used crystal formation as a test of Ca penetration into root hairs of *Dianthus.*

The use of the cells of *Elodea,* in which crystals do not occur normally, was suggested to us by the work of Nadson and Rochline-Gleichgewicht (1927) and Rochline-Gleichgewicht (1930). These authors noted the appearance of CaC_2O_4 crystals in cells of *E. densa, E. canadensis,* and *Pterygophyllum hepaticæfolium* after ultraviolet irradiation, after exposure to radium emanation, and after plasmolysis.

Identification of Crystals

Calcium oxalate was produced, in our experiments, as tetragonal crystals of the trihydrate and the monoclinic crystals of the monohydrate. The former are the most characteristic, as well as the largest and most easily recognized. Large crystals of both types are shown in Fig. 1. In addition to such crystals, sometimes small, needle-like crystals of calcium oxalate are formed. Their habit cannot be determined.

Although such appearances correspond to descriptions of calcium oxalate in the literature, and to our own *in vitro* products, it seemed worth while to verify this. The solubilities of experimentally produced crystals in 5N H_2SO_4 and in 10M acetic acid were compared. The crystals dissolved in the sulphuric, but not in the acetic acid. This behavior is characteristic of calcium oxalate. But the most elegant method for the identification of microscopic crystals is that of refractive index determination.

Such determinations were made for us by Mr. A. W. Postel, of the Geology Department of this University. They demanded the greatest skill and patience, as the largest crystals obtainable in *Elodea* cells are much smaller than those usually found in petrological material.

The indices of refraction were obtained by the immersion method. The crystals were bathed in liquids of known refractive index, until those liquids were found which had the same indices as the crystals; in such liquids all relief between crystal and liquid vanishes. In the present work the Becke line method was used (for description see Chamot and Mason, 1931, Vol. 1). Since the crystals in our cells were anisotropic, the measurements had to be made with the polarizing microscope, as the values of the several indices of the same crystal can be obtained only by placing the vibration planes of the crystal successively in a position parallel to the vibration plane of the lower Nicol prism.

The largest crystals were obtained in leaves plasmolyzed for about thirty minutes in 0.5M sucrose, and deplasmolyzed in tap water. These leaves were dehydrated in ethyl followed by isoamyl alcohol.

The matching liquids were a series of mixtures of isoamyl alcohol and alpha-monochlor-naphthalene (halowax oil), ranging in refractive index from 1.440 to 1.600 in gradations of about 0.005. The liquids were introduced into the dehydrated cell by the " sinking method " used for clearing microscopic preparations (Lee, 1900, p. 79). After the leaf had sunk through a column of a mixture, it was immersed for some time in a fresh quantity of the same liquid. Five-cc. samples were used.

The leaf was now ready for examination with the polarizing microscope. When matches were obtained, the exact refractive indices of the

liquids were determined by means of a goniometer with a hollow prism for the liquid.

Exact values were obtained for the tetragonal crystals. The indices that could be measured came out as follows:

ω	ϵ
1.491	1.540
1.505	
1.499	
1.495	

These figures are in agreement with those given by Wherry and Keenan (1923). The shape of the other crystals, as well as the fact that they had one index of refraction higher and one lower than the highest index of the tetragonal crystals, was taken as sufficient evidence that they were monoclinic crystals of calcium oxalate monohydrate.

The optical properties of our crystals, therefore, identify them as calcium oxalate. Parallel determinations on artificially prepared calcium oxalate monohydrate and trihydrate verified this conclusion.

Action of Stimulating Agents

Agents Used

Stimulating agents may be defined as physical or chemical agents which call forth the characteristic responses of irritable cells. In working on a general theory of stimulation, it is necessary to use those agents which affect the most familiar types of responsive cells, such as muscle, nerve, and unfertilized egg cells. Among such general stimulants are electric shocks, radiations, changes in temperature, mechanical shock, a variety of chemical agents, and osmotic changes. To test Heilbrunn's hypothesis, which was the purpose of this work, cells of *Elodea canadensis* were subjected to all these influences to see whether the latter caused a release of calcium in the cell interior.

EXPLANATION OF PLATE I

Fig. 1. Crystals produced by plasmolysis in 0.5M sucrose and deplasmolysis in pond water. Crystals at lower left tetragonal, others monoclinic.

Fig. 2. Crystals produced by two condenser shocks. Twenty volts, 2 microfarads.

Fig. 3. Deposit of crystals toward anode (left in all figures). Current 0.75 milliampere for 15 seconds.

Fig. 4. Deposit of crystals toward anode, under higher magnification. Same current as in Fig. 2.

Fig. 5. Electrical breakdown of plasmolyzed cells. Normal cell marked by arrow.

Fig. 6. Same field at lower focus, showing crystals which have settled down within the tonoplasts.

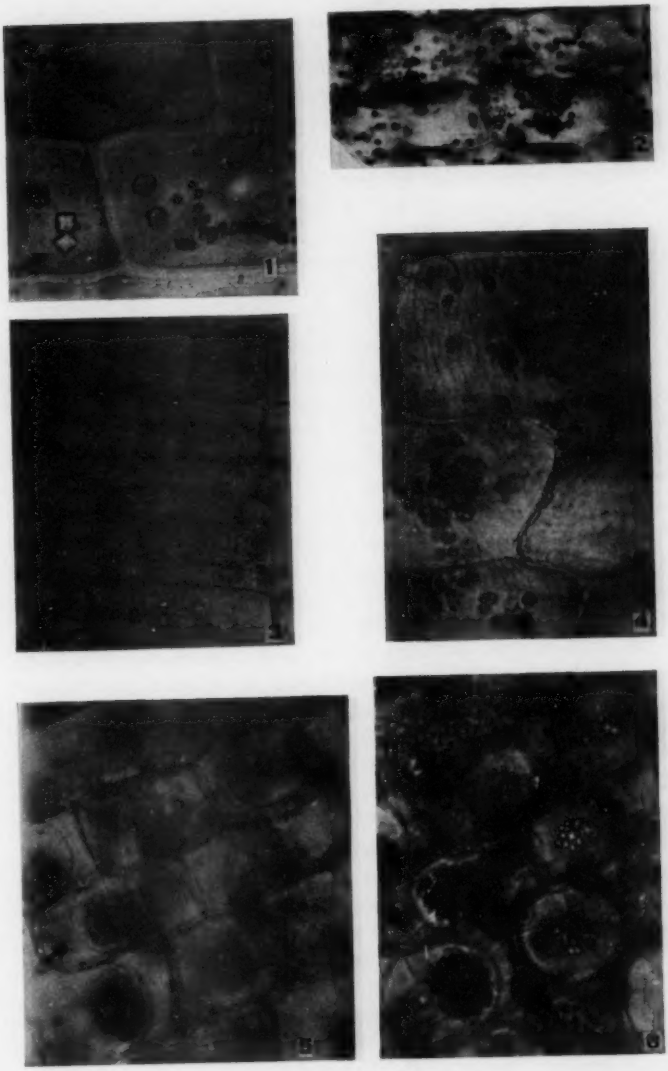

PLATE I

Effect of Electric Current

Condenser Discharges.—In studying electrical stimulation it seemed desirable to use shocks of brief duration in order to minimize such factors as electroendosmosis. A condenser of 2-microfarad capacity was charged from a " B " battery that gave about 20 volts, and discharged through Cu-$CuSO_4$ electrodes of simple design. Glass tubes of 6 mm. bore were filled at their tips with a 3–4 per cent agar gel made up in tap water or 0.1M NaCl. Over the gel was placed saturated $CuSO_4$ solution, and the copper wires were dipped into this.

The *Elodea* leaf was dried on filter paper and placed on a glass slide. The electrode tips were placed a few millimeters apart, because the resistivity of the tissue was found to be so great that effective currents could not be passed through the whole length of the leaf with the apparatus used. When more than one shock was used, the shocks were spaced at intervals of one second or two seconds.

At first, 5–10 shocks were used. These caused the deposit of many crystals in the vacuoles although the protoplasm streamed vigorously, more vigorously if anything, after the shocks. It was soon found that definite production of crystals results from application of a single shock, or two shocks. Cells that had received two shocks are shown in Fig. 2. In general, more shocks produced more crystals. One had to use more than 20 shocks to kill some of the cells.

The crystals were localized at the anodal end of the cell, as Fig. 2 shows. Later they became scattered as a result of streaming motion, but if observed immediately after stimulation, it could clearly be seen that calcium oxalate forms at the anodal end on stimulation.

Continuous Direct Current.—When a 110-volt source of D.C. was used, the current could be passed through the whole leaf, and the cells observed during the passage of the current. The same electrodes were used as in the condenser experiments.

When a current of 1 milliampere is made and instantly broken, the immediate effects are a slight but definite jerking of the protoplasm away from the cathodal end of the cell, and the appearance, very soon, of small crystals of calcium oxalate in the vacuole at the anodal end. If the current is allowed to flow for a second or two, and then broken, the same effects become more pronounced. At the cathodal end the protoplasmic layer thickens markedly and the convex contour of the tonoplast becomes visible. At the anode the protoplasmic layer becomes thin, and the protoplasmic strands in the vacuole are broken, the protoplasm being drawn into the main mass. More crystals appear in the vacuole at the anodal end. If at this stage the cells are treated with 0.33M sucrose,

they plasmolyze, but the protoplasts adhere to the cell wall at the anodal end.

If a larger amount of current is used, a layer of small crystals forms along the extreme anodal boundary of the protoplast, appearing to be contiguous to the cell wall (Figs. 3, 4). A latent period may intervene between the breaking of the current and the appearance of this crystal deposit. The formation of this anodal deposit of crystals marks the beginning of the disintegration of the cell membrane. This first appears toward the anode. With further passage of the current, the disintegration passes in a wave over the surface of the protoplast, from anode to cathode. Occasionally, it has seemed that it went in the reverse direction. As the wave passes, calcium is released. Apparently free oxalate is available at the site of the disintegration, for the wave leaves behind it a wake of calcium oxalate crystals on the surface of the protoplast. The wave passes rather slowly, its speed depending on the current applied. If the current is broken, it stops, and is seen as a tenuous, refractive boundary, the edge, apparently, of a perfectly transparent layer.

The wave reported by Weis (1925) in *Allium cepa* cells may be similar, though from his description we would judge that he was observing a cathodal movement of the tonoplast.

Another phenomenon observed in cells subjected to severe currents was the frequent pulling-in of the protoplast along the sides of the cells, particularly at points where it was convex.

Once a deposit of crystals has formed along the anodal end of the protoplast, it is no longer possible to plasmolyze the cells; they do not recover their semipermeability on standing. The chloroplasts shrink and assume puckered shapes.

To determine the relationship between membrane disintegration and calcium release, cells were plasmolyzed with 0.5M sucrose, and the protoplasts were subjected to the electric current in this medium. Since the conductance is low, most of the current is shunted through the cells, so that small currents will have severe effects.

Currents of 0.5–1 milliampere were used. When the circuit is made and instantly broken, all the protoplasts jerk toward the anode (*cf.* Rubinstein and Uspenskaya, 1934). Slightly more current causes some cells to break down; others are more resistant, and some never break down.

The cells which do break down rupture first at the anode. The plasmalemma breaks, at one point as a rule, but sometimes at several. Protoplasmic granules flow out, and a few calcium oxalate crystals are seen to form outside the protoplast. The tonoplast protrudes through the

point of rupture, enlarging the break in the protoplasmic layer. The clear contents of the vacuole become filled with crystals, beginning at the point of exposure, and a wave of plasmalemma disintegration passes over the surface from anode to cathode, leaving the tonoplast to round up again (Figs. 5, 6). The protoplasmic layer, also broken at the anode, "peels" off from the tonoplast, and is clumped at the cathodal end of the cell. Sometimes the tonoplast is freed in two parts, or fragments break away, becoming small, clear spheres containing crystals.

When the current is allowed to flow for 15–30 seconds, the tonoplasts, too, break down, contracting first, then disintegrating after anodal rupture.

Our experiments did not reveal any natural polarity in the cells of the *Elodea* leaf. The results were the same whether the current was passed from base to tip or vice-versa. If the leaf was placed transversely in the electrical field, crystals were in this case also deposited at the anodal end.

The explanation of the anodal calcium release cannot be discussed at length here. An obvious suggestion is that a local increase in acidity decreases the capacity of the proteins to bind Ca. However, the observations of Kühne (1864), Bethe (1915), Mast (1931), led to the conclusion that plant cells subjected to direct currents become more alkaline at the anode, and acid at the cathode. However, Blinks (1932) attributes the apparent polar change in color in the natural anthocyanin indicators used by the above authors to electrophoresis rather than to change in reaction.

We performed a number of experiments to see whether acid could cause the setting free of Ca and the precipitation of calcium oxalate. Leaves were placed in 0.1N or 0.2N acetate buffers of pH 3.8, 4.2, 4.6, 5.0, 5.8. These external pH values tell nothing about the effect on the intracellular pH, for the acetic acid penetrates rapidly while the sodium acetate penetrates slowly if at all. The leaves in these solutions were examined at intervals up to two hours. Various morphological changes were observed, but in no case did crystals appear. Therefore, the hypothesis that local acid accumulation is responsible for calcium release when direct currents are applied was not verified.

Effect of Oxalate.—The vacuole of the *Elodea* cell contains a fairly high concentration of free oxalate. There is also reason for believing that there is oxalate in the protoplasm (see discussion). Yet, if the observations of Heilbrunn and Daugherty (1933) are of general significance, we must expect that immersing the *Elodea* cell as a whole in an oxalate solution should prevent calcium release. In testing this, leaves were washed in 0.05M sodium oxalate before they were exposed

to electric currents. Since this solution has such a high conductivity as compared to the normal medium of the cells, they were removed from it after a minimum of ten minutes exposure and placed in 0.001M sodium oxalate or in distilled water. The same strengths of electric current were applied to these cells as had been applied to cells not washed in oxalate.

The results were consistent in many experiments. No crystals were produced no matter how long the current was passed. The plasmalemma did disintegrate in a wave beginning at the anode, but even this was not accompanied by crystal production.

Mechanical Stimulation

Mechanical shock is a very general stimulant. Its effects on cells are discussed in a review by Lepeschkin (1936) and by Angerer (1936). The latter, working on *Amœba,* has found that the viscosity changes associated with mechanical agitation are similar to those described by Heilbrunn and Daugherty (1933) for ultraviolet irradiation, and therefore in accord with the hypothesis that calcium release is a primary effect of the stimulation.

Our method of applying the stimulus was very primitive. A leaf was placed between two microscope slides and rapped sharply with heavy forceps. In one experiment in which the leaf was rapped 50 times and examined immediately, more than half of the cells showed very clear crystal formation, most of the crystals being of the needle-like variety. The cells were not killed, but seemed to be streaming at a somewhat more rapid rate than normally. The effect of mechanical stimulation was tried on leaves from many different specimens of *Elodea.* Crystals always could be obtained, although sometimes very many shocks were necessary.

Irradiation with Ultraviolet Light

The valuable paper of Nadson and Rochline-Gleichgewicht on the production of calcium oxalate crystals as a result of ultraviolet irradiation has already been mentioned. There was no difficulty in verifying the observations of these authors. Leaves were irradiated from a Cooper-Hewitt quartz mercury vapor lamp, at a distance from the arc of 25 centimeters. The leaves were exposed for 2.5 minutes, surrounded only by an adhering film of water. On examination within five minutes after treatment, they showed a definite but not profuse precipitation of calcium oxalate in the vacuoles of the cells. Most of

the crystals were tetragonal. Three minutes irradiation caused a much heavier precipitation.

At the same time leaves were irradiated after having been immersed for 15 minutes in 0.05M sodium oxalate. In these leaves even 10 minutes after irradiation there was no sign of calcium oxalate in the vacuoles of the cells.

Therefore, just as in the case of electrical stimulation, treatment of the outside of the cell with oxalate prevents the release of calcium ion in the interior.

Heat

Heat stimulates many living systems; it is listed by Bethe (1915) among the general protoplasmic stimulants. In our preliminary experiments *Elodea* leaves were immersed in water at 100° C. This treatment killed the cells without producing any crystals.

We then tried heat treatments of the same order of magnitude that have been found to stimulate other cells, particularly egg cells. Heating at 35° for more than an hour caused no crystal production. At 40° many crystals, a mixture of fine needles and large tetragonal crystals, were formed after 60 minutes exposure. At 45° exposures of 20 minutes duration produced many crystals in all the cells. These cells plasmolyzed normally, after the heat treatment, in 0.3M sucrose. At 50°, 8 to 10 minutes exposure caused the production of crystals without destroying the ability of the cells to plasmolyse in the 0.3M sucrose solutions. At 60°, however, all the cells died after exposure of a minute or two—and no crystals were produced!

To determine the effect of oxalate on the reaction of the cells to heat, leaves were washed in 0.05M sodium oxalate, and subjected to the heat treatments described above while they were still in the sodium oxalate solution. No crystals were produced.

We attempted to decide whether the effects described above were due to the absolute heat treatments we used, or whether temperature change was the causal factor. We studied the effect of rapid cooling by immersion in an ice-water mixture. No crystals were produced by 10-, 20- and 60-minute immersions. When the cooled cells were placed in water at room temperature, no crystals were produced. Therefore, high temperatures themselves were responsible for crystal production by heating.

Osmotic Changes

Bethe, in the list of general stimulants cited above, includes osmotic influences; hypertonic solutions particularly are stimulating agents.

If cells are plasmolyzed in 0.5M sucrose and deplasmolyzed in pond water, they are then found to have crystals in their vacuoles. When the process is followed under the microscope, it is observed that the crystals do not appear until deplasmolysis begins. The first effect observed, upon addition of the deplasmolyzing agent, is a strong contraction of the tonoplast. This is observed, even after complete, convex plasmolysis. It is strikingly rapid and vigorous, and even after harsh plasmolysis in 1.3M $CaCl_2$ deplasmolysis is preceded by further contraction.

After strong plasmolysis, e.g., after 10 minutes or more in 0.5M sucrose, deplasmolysis leads to the death of most of the cells of *Elodea* leaves. The protoplasm expands in a series of slight jerks, which are due to the rupture, at various points, of the plasmalemma. The vacuoles of such cells acquire many crystals, even when a deplasmolyzing agent such as 0.25M sucrose or distilled water, lacking any calcium, is used. In this type of fatal plasmolysis, the tonoplast breaks down as well as the plasmalemma.

When weaker plasmolysis is used, such as follows exposure for less than 10 minutes to 0.5M sucrose, or 10–15 minutes in 0.33M sucrose, deplasmolysis is smooth and the cell may be replasmolyzed. Less crystals are obtained in these cells, but there is always a definite precipitate. Sometimes these crystals may adhere to the vacuole wall and be carried about as the protoplasm streams. When such cells are bathed in 0.05M $CaCl_2$, no further precipitation of calcium oxalate is observed.

Many experiments on the effects of very weak plasmolysis were performed. When cells are treated with a 0.2M NaCl solution, there is a preliminary stage, lasting about three minutes, in which the vacuole shrinks markedly, but the plasmalemma does not separate from the cell wall. If a hypotonic solution is added as soon as this preliminary stage of plasmolysis is established, the tonoplast expands and the cell resumes its normal appearance without the production of any crystals. If, however, 2–3 minutes are allowed to elapse before the 0.2M NaCl is changed for pond water or any other hypotonic medium (the exchange is effected under the microscope simply by drawing the new medium under the coverslip with strips of filter paper), the protoplast, which had not yet contracted in the hypertonic medium, separates from the cell wall, and within a few seconds expands again. In cells in which this transient plasmolysis occurs, crystals are formed. It seems, therefore, that actual shrinkage of the protoplast is necessary to cause calcium release after treatment with hypertonic solutions. It is immaterial whether this plasmolysis is produced while the cell is in the hypertonic medium, or as a result of the " contraction " that occurs on transfer from the hypertonic medium to the hypotonic.

Other experiments on the effect of mild plasmolysis were performed with the balanced mixture (0.18M NaCl plus 0.005M $CaCl_2$) as plasmolyzing agent, and the same mixture diluted to half strength as deplasmolyzing agent. The time of exposure to the hypertonic solution was varied. The cells showed vigorous protoplasmic streaming after deplasmolysis, and the vacuoles contained crystals, usually small ones but sometimes numerous. We were unable to correlate time of plasmolysis with the amount of precipitate, although a quantitative method might reveal such a correlation. The precipitate could not be increased by washing the cells with a 0.05M $CaCl_2$ solution, except in those few cells that had been broken even by the mild treatment.

These experiments answer the objection that the crystal formation may be ascribed to penetration of calcium from the external medium, after rupture of the plasmalemma by our treatment. First, in the experiments with mild plasmolysis there is no indication of membrane rupture. Second, although after strong plasmolysis more crystals can be obtained when 0.05M $CaCl_2$ is used as deplasmolyzing agent, very many crystals are obtained when distilled water, 0.1M NaCl, or 0.25M sucrose are used. Finally, when 0.05M sodium oxalate was used as deplasmolyzing agent, fewer crystals were produced, but there was a definite, sometimes an abundant, precipitate.

The effect of oxalate on the calcium release in plasmolysis-deplasmolysis was investigated. Cells were immersed for various periods in a solution of 0.5M sucrose in 0.1M sodium oxalate. With prolonged immersion—30 minutes or more—the cells gradually undergo "systrophy," in which the main mass of cytoplasm and cytoplasmic inclusions flows together at one part of the cell, while the remainder appears as a thin layer around the now-visible tonoplast (*cf.* Küster, 1929, p. 172). If the cells at any time after plasmolysis in this solution are treated with distilled water, they deplasmolyze. Typically no crystals are produced.

In this part of the work two types of experiments are to be distinguished: (1) experiments in which plasmolysis is brief, or in which the weakly hypertonic solution 0.33M sucrose in 0.1M $Na_2C_2O_4$ is used; and (2) experiments in which strong plasmolysis, leading to systrophy, is obtained in 0.5M sucrose in 0.1M sodium oxalate. In the first group of experiments, deplasmolysis was smooth and the cells were not killed; they could be replasmolyzed. A number of deplasmolyzing agents were tried: distilled water, pond water, 0.1M NaCl plus 0.005M $CaCl_2$, 0.05M sodium oxalate. No crystals were obtained with any of these. Therefore the results are in accord with those obtained with electrical, thermal,

ultraviolet stimulation: washing in oxalate prevents the release of calcium which otherwise takes place.

In the second group of experiments, in which deplasmolysis of the systrophied cells does involve rupture, no crystals were obtained when distilled water or 0.05M sodium oxalate was used, but crystals were obtained when pond water or 0.005M $CaCl_2$ was used. This result is enlightening, for it shows that the tonoplast is permeable to Ca; observation showed that the crystals were formed as soon as the plasmalemma ruptured.

To test whether the oxalate actually removes the Ca from the surface, or whether the presence of the oxalate ion itself is responsible for the prevention of crystal formation, some cells were first plasmolyzed in 0.5M sucrose in 0.1M sodium oxalate, then washed in 0.5M sucrose alone, then deplasmolyzed in 0.25M sucrose or in distilled water. No crystals were produced. Therefore, the oxalate does remove the Ca or whatever is responsible for the Ca release, from the surface.

If it is Ca that is removed from the surface by oxalate, one might be able to restore it, and thus to reverse the effect of the oxalate. Leaves treated with 0.05M sodium oxalate for 10 minutes—more than enough to prevent crystal formation, were washed in distilled water. Some of them were then placed for ten minutes in pond water or 0.02 $CaCl_2$, others not. Both groups were then plasmolyzed in 0.05M sucrose and deplasmolyzed in distilled water. The cells that had been treated with pond water or $CaCl_2$ formed crystals on deplasmolysis—the effect of the oxalate had been reversed. The other leaves showed no crystals, or many fewer crystals. Therefore, oxalate does indeed act by removing Ca from the surface, and this Ca may easily be replaced.

One interesting observation made in this part of the work was that violent deplasmolysis after plasmolysis in oxalate-containing solutions did not destroy the tonoplast. Especially after systrophy, completely isolated tonoplasts could be obtained.

Substitution of Citrate for Oxalate

In the experiments on the prevention of calcium release, oxalate was used because it had been used by the previous authors whose ideas we were testing. Similar experiments were later performed in which citrate was substituted for oxalate. Although citrate does not precipitate Ca, it binds it in a non-ionized complex. Furthermore, the pK of this $CaCit^-$ is so large, that at high citrate concentrations the citrate should bind Ca at the expense of proteins (*cf.* McLean and Hastings, 1935).

Our tests were made with electrical stimulation, and with plasmolysis-

deplasmolysis treatment. Leaves were washed in 0.04M sodium citrate for 10 minutes, then rinsed in distilled water and subjected to direct currents of about 1 milliampere. No crystals were formed, although the usual deposits were obtained in cells treated with distilled water alone.

The only visible effect of immersion in sodium citrate was a tendency of the chloroplasts to flow together, which was reversed on their return to pond water.

In studying the effect of citrate on plasmolysis-deplasmolysis, the following procedure was used. Leaves were washed in 0.035M sodium citrate for 10 minutes, then rinsed in distilled water and plasmolyzed in 0.05M sucrose. On deplasmolysis with distilled water no crystals were formed. In control leaves, treated similarly except for the exposure to citrate, the usual precipitate of calcium oxalate was obtained. It must be remarked here that this experiment is much clearer than the corresponding one with oxalate; in the latter there are occasional doubtful cases and the presence of crystals on the outside of the cells makes judgment difficult.

The reversibility of the citrate effect was also studied. Leaves immersed in citrate for 10 minutes were restored to pond water or to 0.02M $CaCl_2$ for 10 minutes. They were then plasmolyzed in 0.05M sucrose and deplasmolyzed in distilled water. Crystals were obtained in these leaves; in greater abundance in the leaves that had been treated with the pure Ca salt solution. Tests with the electric current also showed that the effect of the citrate could be reversed by treatment with pond water or calcium solution.

The Effect of Isotonic Salt Solutions

Certain isotonic salt solutions are considered to be stimulating agents. Sodium salt solutions in particular cause muscles to twitch, and favor the action of other stimulants on *Arbacia* eggs. One of us (*cf.* Heilbrunn, Mazia, and Steinbach, 1934) has shown that in the latter case, isotonic NaCl caused an increase in the free Ca concentration of the cell interior.

Elodea leaves were washed and allowed to stand in 0.13M NaCl, 0.13M KCl, and 0.08M $CaCl_2$. They were examined at various times over 24 hours. The cells in NaCl stopped streaming first; at 18 hours the protoplasm was barely moving. At 18 hours, in the $CaCl_2$ solution, the protoplasm was streaming without carrying the chloroplasts, while in KCl the chloroplasts were in motion. No crystals were produced in any of these cells. The experiment was repeated a number of times; in no case was calcium oxalate precipitation observed.

Other Toxic Agents

Having found effective so many agents which, although stimulants, are also injurious, it is necessary to show that injurious agents which are not considered stimulants do not have the same effects as stimulants.

A number of such agents were tried. All of them caused the death of the cells without the production of crystals. They will simply be listed:

1. Ninety-five per cent ethyl alcohol.
2. Cold.
   a. Zero degrees centigrade.
   b. Temperature of solid CO_2.
3. Heat.
   a. Seventy degrees centigrade.
   b. One hundred degrees centigrade.
4. NH_4OH—0.01M.
5. $CuSO_4$—0.01M.

The Effect of Anesthetics

If the release of Ca ion is a general concomitant of the action of stimulants on cells, it is important to know whether anesthetics can prevent this release.

The anesthetics used were ethyl ether, chloral hydrate, and ethyl urethane. Their effect on the response to electrical stimulation and plasmolysis-deplasmolysis was investigated.

Leaves were placed in stoppered test tubes containing 1, 2, 3, 4, and 5 per cent solutions of ethyl ether in distilled water. They were removed after various times of immersion, and their reaction to currents of 1 milliampere was observed. In 1 per cent ether, even after 40 minutes immersion, the electric current caused the formation of crystals to the same extent as in the controls. The same result was obtained in 2 per cent and 3 per cent solutions. In 4 per cent ether the cells reacted like the controls for 20 minutes, then they appeared coagulated and no crystals were produced. Subsequent immersion in pond water for 30 minutes did not restore their responsiveness to the direct current.

More severe treatment, such as 30 minutes in 5 per cent ether, itself caused the production of small tetragonal crystals in most of the cells. The cells were dead; they could not be plasmolyzed and the amount of calcium oxalate could not be increased by applying electric currents.

The sequence of events could be more easily studied in the chloral hydrate experiments. Ten minutes in a 2 per cent solution did not prevent crystal formation. Longer treatment with this solution (13–14 minutes) caused the formation of large crystals, but passage of the

electric current at this stage resulted in the formation of more crystals, concentrated at the anodal end. Still longer treatment with the anesthetic caused crystal formation accompanied by the death of the cells; subsequent treatment with the electric current produced no more crystals. A considerable number of experiments with chloral hydrate established the fact that severe treatments—use of solutions stronger than 2 per cent—cause the formation of large crystals, associated with the rapid death of the cell.

In the study of the effects of anesthetics on plasmolysis-deplasmolysis, the anesthetic was dissolved in the plasmolyzing agent. For example, cells were plasmolyzed in 0.2M NaCl containing 2 per cent ethyl urethane, and deplasmolyzed in pond water. Crystals were produced to the same extent as in the controls. The cells were exposed for as long as 30 minutes to the anesthetic, without effect.

The theoretical implications of these results will be considered in the general discussion.

Discussion

Our results seem to us to uphold the hypothesis that when cells are stimulated, the free Ca concentration within the cell increases. We have shown the applicability of this hypothesis to the *Elodea* cell in its reaction to the most important stimulating agents. More complete verification of the hypothesis would depend on similar studies on widely different types of plant and animal cells.

However, the interpretation of the experimental production of calcium oxalate in plant cells can be examined more closely. The bulk of the free oxalate is in the vacuole; some may be in the protoplasm. Only a trace of Ca could be in a dissolved state in the vacuole unless the pH is 4 or less, at which reaction calcium oxalate is difficult to precipitate. Therefore, in our experiments, the Ca must come from the rest of the cell or from the surrounding medium. The latter possibility is eliminated by experimental results, discussed on p. 316.

Granted that the Ca comes from the protoplasm, it remains to be shown why calcium oxalate does not precipitate in the vacuole until the cell is subjected to the action of stimulating agents. There are two possibilities: (1) The vacuole membrane is impermeable to Ca, but becomes permeable at the moment of stimulation. (2) There is no considerable concentration of free Ca in the protoplasm, but bound Ca becomes free when stimulating agents are applied.

The first alternative faces two difficulties. In experiments in which the plasmalemma ruptures in Ca-containing solutions the vacuole immediately becomes packed with crystals. The vacuole membrane, there-

fore, seems to be very permeable to Ca, although this experiment does not prove certainly that this membrane is permeable when the plasmalemma is intact.

A second difficulty is that the cytoplasm seems to contain some free oxalate, which would bind any pre-existing free Ca. It could be shown experimentally that isolated tonoplasts are permeable to oxalate. If they are washed for an hour in distilled water, and Ca is added, no crystals are formed in the tonoplasts, although they can be formed before the washing. The observation recorded on p. 311, that on electrical disintegration of the plasmalemma in sugar solution some crystals are formed outside the vacuole, also supports the idea that the protoplasm contains oxalate. Finally, the oxalate must originally form in the cytoplasm; and would be precipitated as it formed if free Ca were present.

These facts support the second alternative, that the Ca which precipitates the oxalate in our experiments is released from a bound state on stimulation. Here the experiments with oxalate (and citrate) must be considered. The plasmalemma is relatively impermeable to sodium oxalate; cells plasmolyze in hypertonic solutions of this salt and do not deplasmolyze in a short time. Therefore, whenever cells are washed in oxalate, only the outer layer is affected. This is better proved by the fact that the effect of oxalate and citrate can be reversed by solutions containing Ca; if there is doubt about the relative impermeability of the cell to these ions, there is at least less doubt (considering the observations recorded on p. 318) about its relative impermeability to Ca.

If oxalate and citrate, in removing Ca from the outer layer of the cell, prevent the precipitation of calcium oxalate by agents which normally cause it, it follows that it is just this Ca in the outer surface that becomes free and forms the crystals in the vacuole. Oxalate and citrate can remove Ca from its combination with proteins. In ordinary analysis all the Ca in blood serum is precipitated by oxalate, although 50 per cent of it is bound. McLean and Hastings, cited above, have shown the same to be true for citrate.

In this discussion we may not have considered all the possibilities. The argument as it stands, however, indicates that when stimulants act on the *Elodea* cell, they act primarily on the non-diffusible calcium complexes in the cell exterior, releasing free Ca into the interior, some of which precipitates with the free oxalate that is present in the cell interior. This is the argument advanced by Heilbrunn and Daugherty (1933), as an interpretation of the viscosity changes in the exterior and interior protoplasm of *Amœba* under the influence of ultraviolet rays. Our direct results, therefore, support their contentions so far as this one reaction—Ca release—is concerned.

In considering our results in relation to the Heilbrunn theory, the results of the experiments with anesthetics must be explained. Fortunately, Daugherty (1936; in press) now presents evidence from viscosity measurements that is in accord with our evidence, and yet predictable from the Heilbrunn theory. She has found that the viscosity changes which she interprets on the basis of Ca changes are not affected by anesthetics. What is affected is the "clotting" reaction that normally results from the Ca release; Heilbrunn (1934) had already shown that this reaction was prevented by anesthetics in the presence of an adequate amount of free Ca.

Summary

We have attempted in a general way to test the hypothesis that a primary action of stimulants on cells is to cause an increase in the free Ca concentration within the cell.

1. The formation of crystals of calcium oxalate in the *Elodea* cell may be used as an intracellular method for detecting free Ca.

2. These crystals can be positively identified as calcium oxalate.

3. Condenser discharges and continuous direct currents cause the formation of crystals. The crystals are always formed at the anodal end of the cell.

4. Previous washing in solutions of oxalates and citrates prevents the formation of these crystals.

5. Ultraviolet irradiation causes the formation of calcium oxalate crystals. Previous washing in oxalate prevents this.

6. Mechanical stimulation causes the formation of crystals.

7. Heat treatment within the temperature range 40°–55° causes the formation of crystals. Oxalate prevents this.

8. Plasmolysis followed by deplasmolysis causes the formation of crystals. Oxalate and citrate prevent it.

9. The effect of the oxalate and citrate may be reversed by subsequent immersion in Ca-containing solutions.

10. Isotonic salt solutions, and a series of toxic agents, do not cause the formation of crystals.

11. Anesthetics in non-lethal doses do not prevent the formation of crystals by electric currents or osmotic changes.

12. The results are shown to be consistent with the idea that when stimulants act on these cells, they cause a release of Ca from combinations located in the periphery of the cells.

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OBSERVATIONS ON THE MULTIPLICATION OF BACTERIA IN DIFFERENT VOLUMES OF STORED SEA WATER AND THE INFLUENCE OF OXYGEN TENSION AND SOLID SURFACES

CLAUDE E. ZOBELL AND D. QUENTIN ANDERSON

(From the Scripps Institution of Oceanography, University of California, La Jolla, California)

Introduction

The storage of raw sea water in the laboratory is usually accompanied by increased bacterial activity. This may be manifested by the evolution of carbon dioxide, increase in the hydrogen-ion concentration, oxygen consumption, ammonia production, nitrate reduction, or by bacterial multiplication. Waksman and Carey (1935*a*) report that the bacterial population of raw sea water increases from a few hundred bacteria per cc. to millions per cc. following a few days storage in the laboratory. They (1935*b*) attribute this great increase primarily to the dying out of Protozoa and other marine animals which devour bacteria and to the modification of "certain controlling factors injurious to free bacterial development." Keys, Christensen, and Krogh (1935), who observed similar changes, believe that the activity of bacteria in the sea is limited by the "extreme stability of the ocean as a chemical and physical factor."

In their preliminary studies on sampling methods, ZoBell and Feltham (1934) observed a great increase in the number of bacteria in samples of stored sea water even at refrigeration temperatures and also a diminution in the number of varieties. These changes were found to occur less rapidly in large samples than in small ones. The effect of volume on the multiplication of bacteria in stored water was recognized by both Harvey (1925) and Föyn and Gran (1928), who mention the work of Whipple on stored fresh water. Whipple (1901) noted that the smaller the volume of fresh water, the greater is the rate of bacterial multiplication in it. After 24 hours of storage he found 300 bacteria per cc. in a gallon, 7,020 per cc. in a pint, and 41,400 per cc. in 2 ounces of a representative water sample having an initial count of 77 bacteria per cc.

While it is impracticable either to collect or to store samples of sea

water which are large enough to prevent for more than a few hours undesirable changes in its bacterial and chemical composition, the fact that the volume seems to influence the activity of bacteria may be of significance. For example, in one of their experiments on its organic-matter content Keys, Christensen, and Krogh (1935) ultrafiltered small amounts of sea water free of all particulate and colloidal organic matter. After its inoculation with normal flora there was just as much bacterial multiplication in it as in ordinary filtered sea water. Is it possible that the results were influenced by the smallness of the volume of ultra-filtered sea water? Other questions arise: Is the great increase in the bacterial population which accompanies the storage of sea water in any way related to its confinement to a small volume, and if so, to what extent and how? Also, if bacterial activity is influenced by volume, to what extent do controlled test-tube experiments indicate what is actually occurring in the ocean? In summarizing the factors which influence bacterial growth curves Rahn (1932) concludes that the volume of the media has no influence on the multiplication of bacteria. Woodruff (1911) and Greenleaf (1926) report that certain Protozoa multiply faster in large volumes than in small ones while Robertson (1922) found the antithesis of this.

Experimental Methods

Sea water was collected in glass battery jars from the end of the Scripps Institution pier which extends 1,000 feet seaward where the mean depth of the water is about 20 feet. Despite its proximity to land, the water is chemically and biologically quite typical of marine conditions because of the oceanic circulation and the freedom from terrestrial drainage. The water was siphoned into a Buchner 4G sinter-glass filter and filtered by gravity into a 10-gallon bottle. The filtration was merely to remove particulate matter and to permit the oxygen tension to come to equilibrium with the atmosphere. After thoroughly mixing the water by vigorous shaking to insure greater uniformity in its composition, it was siphoned into different sizes of Pyrex bottles or flasks. Some were filled to capacity and glass-stoppered and others were only partly filled, thus leaving the water in contact with air. Chemically clean, sterilized apparatus and aseptic technique were used throughout the experiments.

The bacterial population of the water was determined by plating procedures immediately after its collection from the sea, again after it had been distributed to bottles of different sizes and at periodic intervals thereafter. Two or more dilutions of each sample were plated in duplicate. Sterile sea water was used as the diluent. The following nutrient

agar has proved to yield larger plate counts than any of several other formulæ which have been tried:

Proteose peptone	2.0 grams
Bacto peptone	3.0 grams
Beef extract	2.0 grams
$FeCl_3 \cdot 6H_2O$	0.2 gram
Bacto agar	12.0 grams
Sea water	1,000.0 cc.

After dissolving the agar by steaming, the reaction was adjusted to pH 7.8 with 1/N NaOH and the medium was clarified by filtering it through glass wool. The oxygen content of the sea water was estimated with Winkler's reagents.

Results

In the first series of experiments glass-stoppered Pyrex bottles having similar shapes, proportional dimensions and different capacities were

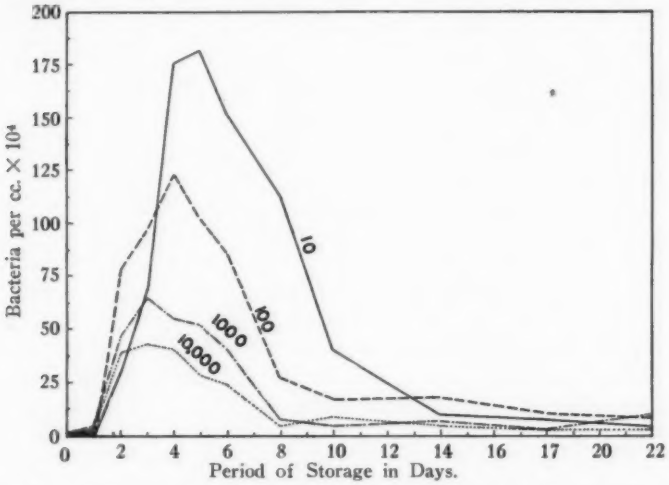

Fig. 1. Influence of volume on the multiplication of bacteria in stored sea water. Growth curve in 10 cc. represented by solid line, 100 cc. by dashed line, 1,000 cc. by dot-dashed line and 10,000 cc. by dotted line.

filled about five-sixths full with 10, 100, 1,000, and 10,000 cc. of raw sea water. The air in the upper one-sixth of the bottles provided for the saturation of the water with oxygen and it permitted more thorough mixing when the water was shaken at the time of sampling. The bottled water was stored in a dark constant temperature room at 16° C. Samples were withdrawn daily for plate counts. Fig. 1 shows graphically

the number of bacteria which were found per cc. of water after different periods of storage in bottles of different sizes.

This experiment has been repeated several times under a variety of conditions. Almost invariably the densest bacterial populations appear in the smallest volumes of sea water. Table I summarizes the maximum plate counts found in four different volumes of three different samples of sea water. It is impossible to duplicate results in the usual sense of the term because each different sample of sea water varies in chemical and biological composition. The initial plate counts of the water collected for these experiments during the last four years has varied from less than 100 bacteria per cc. to nearly 30,000 per cc. Of greater importance is the organic-matter content of sea water which fluctuates in quality and quantity (Bond, 1933). Waksman and Carey (1935*b*) have

TABLE I

Maximum number of bacteria found per cc. of sea water after storage at 16° C. in glass-stoppered Pyrex bottles of different sizes.

Sample No.	86	149	165
Date collected	9/14/35	1/9/36	2/6/36
Initial count per cc.	930	470	2,570
Volume of sea water	Plate count per cc. following storage		
10 cc.	1,050,000	1,960,000	1,810,000
100 cc.	680,000	1,240,000	1,070,000
1,000 cc.	251,000	645,000	615,000
10,000 cc.	164,000	255,000	340,000

shown that the amount and specific nature of organic substances control the abundance of the bacterial population developing in sea water.

As indicated by Fig. 1, during the *maximum stationary phase* of the growth curves (Winslow, 1928) there may be twice as many bacteria per cc. in small volumes as in volumes ten times as large. It requires from three to six days storage at 16° C. for the maximum to be attained. The densest bacterial populations which appear in the smallest volumes of stored water require the longest periods of incubation. As a general rule, the growth curves in the smaller volumes are still in the phase of *logarithmic increase* while the bacterial populations in the larger volumes are declining. Although there is evidence of multiplication within eight hours after the water is bottled, little or no difference is found in the density of the bacterial populations in the different volumes during the first two days. During the *phase of decrease* the bacterial populations in the smaller volumes continue to exceed those in the larger volumes

until the *phase of readjustment* is reached 8 to 18 days after the sea water was first stored at 16° C. During the latter phase the effect of volume on the plate counts disappears entirely as the bacterial populations fluctuate irregularly from a few thousand to over a hundred thousand bacteria per cc. At least a few thousand bacteria per cc. can be demonstrated in any volume of stored sea water for several weeks. After four years storage at 2 to 6° C. in a 100-cc. glass-stoppered bottle there were 209,000 bacteria per cc. of sea water.

In some experiments 1.0 cc. of sea water was stored in test tubes. Each successive day two of these were analyzed by washing them out several times with a 99 cc. dilution water blank. The wash water was appropriately diluted and plated. Also 1.0 cc. volumes were stored in 1.0 cc. pipettes which were filled to the mark, and the water retained by forcing the end of the pipette into a rubber stopper having a small hole bored halfway through it. These were similarly analyzed in duplicate by delivering the contents into 99-cc. water blanks and then washing the pipettes by filling and emptying several times. Table II shows the

TABLE II

Number of bacteria per cc. of sea water following storage at 16° C. of different volumes in various receptacles.

Storage period	1.0 cc. in pipettes	1.0 cc. in test tubes	10 cc. in test tubes	100 cc. in bottles
Start	280	280	280	280
2 days	131,000	128,000	115,000	275,000
3 days	1,210,000	1,340,000	570,000	580,000
4 days	1,230,000	1,410,000	955,000	640,000
5 days	1,510,000	1,895,000	640,000	420,000
6 days	1,300,000	1,510,000	280,000	250,000

average number of bacteria found under both conditions of storage on each day, and for comparison the corresponding plate counts in 10 cc. and 100 cc. volumes of identical sea water are also given. It will be observed that again the smallest volume of sea water supported the largest bacterial populations. Following the storage of some 1.0 cc. samples of sea water, more than 4,000,000 bacteria have been demonstrated by plate counts whereas the maximum bacterial populations in 10-liter samples of the water have been only 2 to 5 per cent as much. The same sea water stored in the interstitial spaces of glass beads or sand which may be regarded as multiple minute volumes supports bacterial populations exceeding ten million per cc.

Inasmuch as it is quite obvious that in small volumes of water the temperature would change more rapidly than in large volumes, precau-

tions were taken to maintain the different volumes of water at the same temperature. First, all of the water in the 10-gallon reservoir was brought to the desired temperature. The change was usually slight because the temperature of the sea water which was collected for most of the experiments was 13 to 16° and it was stored in a constant temperature room at 16°. In order to avoid thermic disturbances, in some experiments the sea water was stored in a water bath at exactly the same temperature as when collected. Later, receptacles of different capacities were filled with filtered sea water on the end of the pier and these were suspended in the sea, 4 to 5 meters below the surface. The bottles were hauled upon the pier each day to permit sampling. Again many more bacteria per cc. were found in the small bottles than in the larger bottles just as was found in the laboratory. Finally, bottles full of sea water were submerged in the sea stoppered with a diatomaceous earth filter candle in a rubber stopper. In other bottles the mouth was narrowed by stoppering with a one-hole rubber stopper in which was inserted a 5 mm. bore glass tube bent at a sharp angle. Under both conditions the bacterial populations in 100 cc. bottles increased to nearly a million per cc. and in the 10-liter carboy to over a hundred thousand bacteria per cc., whereas the sea water *in situ* in which the bottles were submerged contained less than a thousand bacteria per cc.

When sea water is analyzed immediately after collection, from 25 to 35 different species of bacteria can be recognized by a careful scrutinization of their colonial characteristics. Actually 96 different species of marine bacteria have been isolated and culturally, morphologically and physiologically characterized. Almost before there is evidence of multiplication in stored sea water, the number of species begins to decrease until at the time the *maximum stationary phase* in the growth curve is reached, only nine or ten different species are detectable on the plates. Quantitative data cannot be given, but it is our impression that there are usually fewer predominating species in the smaller volumes than in the larger volumes of stored sea water. That many species disappear during storage and do not merely escape detection by being outnumbered by others which multiply rapidly is indicated by the fact that during the *readjustment phase* when the populations have decreased to a few thousand bacteria per cc., only four or five species can be recognized on the plates regardless of the volume.

Effect of Oxygen

Rahn (1932) states that with the same organism in the same medium the final amount of growth " will be proportional to the volume of the medium unless the volume has had an influence upon the rate of oxygen

penetration into the medium." Whipple (1901) attributed the more rapid multiplication of bacteria in small samples of fresh water to the greater oxygen content of the water. However, in his experiments Whipple added 0.0005 per cent peptone to the water samples to hasten bacterial multiplication, and the additional peptone would increase the oxygen consumption. Likewise Rahn's conclusions are based upon results obtained in media rich in organic matter in which there would be appreciable oxygen consumption. There is very little organic matter in sea water. According to Krogh (1931) it contains less than 10 mgm. of organic matter per liter, much of which cannot be utilized by bacteria. Waksman and Carey (1935*a*) as well as Keys, Christensen, and Krogh (1935) find that bacterial activity is arrested by the depletion of available organic matter in stored sea water before half of the dissolved oxygen is consumed.

TABLE III

The initial and maximum plate counts per cc. of sea water stored in partly filled bottles of different sizes and the oxygen content of similar water in open bottles after 7 days quiescent storage at 16° C.

Volume of sea water	Plate count per cc.		Oxygen in cc. per liter	
	Initial	Maximum	Initial	After 7 days
cc.			*cc.*	*cc.*
100	460	1,360,000	5.46	5.14
1,000	460	670,000	5.46	5.39
10,000	460	485,000	5.46	5.38

Those who have criticized our paradoxical observations that small volumes of stored sea water support larger bacterial populations than large volumes suggest that the explanation will most likely be found in the difference in the oxygen content of the water. Therefore this factor has been carefully considered from various angles.

In bottles which were only partly filled with sea water it was found that the contact with the air plus the daily shaking at the time the samples were taken kept the water virtually saturated with oxygen. The oxygen content of the water under these conditions was decreased only slightly even when not shaken. This is illustrated by the data in Table III, which give the maximum bacterial populations which were attained during the storage of different volumes of sea water and the oxygen content of similar water after 7 days quiescent storage.

In the next series of experiments the bottles were filled full, glass-stoppered and submerged in a water bath at 16° C. Duplicate 100 cc. and one-liter bottles (actually somewhat more than 120 and 1,200 cc.

respectively) were analyzed each day for their bacterial and oxygen content. Samples were withdrawn from the 10,000 cc. bottle each day by means of a series of siphons and traps which permitted the withdrawn samples to be replaced by similar sea water from another receptacle without coming into contact with air. The pertinent data of a representative experiment are summarized in Table IV. In every case the oxygen consumption per unit of water was greatest in the smallest volumes in which were found the largest bacterial populations. This would seem to indicate that the oxygen content of the water does not account for the increased bacterial activity in small volumes of sea water.

It may be of interest to note that oxygen consumption in any volume of stored sea water parallels the growth curves until the *maximum stationary phase* is reached. However, during the period of *logarithmic decrease,* oxygen consumption is not retarded but continues in a straight line at the same rate as it does during the *logarithmic phase* of growth. This is illustrated by Fig. 2 which gives the growth and oxygen-consumption curves for 100 and 1,000 cc. of stored sea water. Although there were approximately the same number of viable bacteria per cc. in both volumes as demonstrated by plate counts after the tenth day, twice as much oxygen was consumed per unit of water in the 100-cc. receptacle as in the 1,000-cc. receptacle. This may indicate that there are many more bacteria which are respiring than the number which can be demonstrated by plating procedures.

The oxygen-consumption curves do not continue as straight lines until all the oxygen is depleted, as may be inferred from Fig. 2. Instead the curves start to flatten out between the fifteenth and twenty-first days, and oxygen is consumed only very slowly after 28 days storage at 16° C. Oxygen consumption in liter samples continues at a slower rate and for longer periods of time than in 100-cc. samples.

TABLE IV

The maximum plate counts per cc. of sea water stored in completely filled glass-stoppered bottles of different capacities and the oxygen content of the water after 18 days storage at 16° C.

Volume of sea water	Plate count per cc.		Oxygen per liter		Oxygen consumed per liter
	Initial	Maximum	Initial	18 days	
cc.			cc.	cc.	cc.
100	276	1,010,000	5.40	2.87	2.53
1,000	276	580,000	5.40	4.09	1.31
10,000	276	370,000	5.40	4.43	0.97

Oxygen consumption in 10-liter samples has not been followed for more than three weeks. Regardless of the volume of the samples, oxygen is not depleted from sea water which is initially saturated (5.0 to 5.8 cc. oxygen per liter) unless utilizable organic matter is added. Respiration as indicated by oxygen consumption virtually ceases while there is still between 2 and 3 cc. of oxygen per liter in solution. However, upon the addition of only small amounts of organic matter, the oxygen content is rapidly reduced to zero in any volume of sea water from which atmospheric oxygen is excluded. Strips of filter paper and even agar-agar suffice. It has been shown by ZoBell and Anderson (1936) that bac-

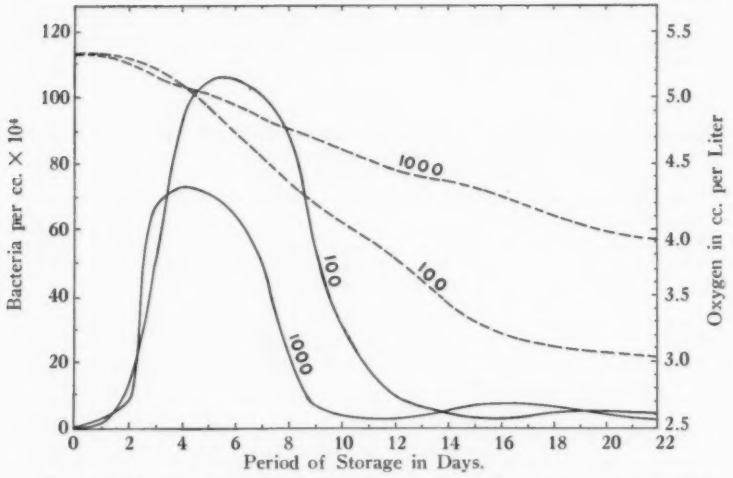

FIG. 2. Influence of volume on oxygen consumption in stored sea water. Solid lines represent growth curves and dashed lines oxygen consumption in 100 and 1,000 cc. respectively.

teria not only deplete the oxygen from marine bottom deposits but that they actually create an oxygen deficit. As will be discussed below, the volume effects disappear when more than a few milligrams of utilizable organic matter per liter is added to the sea water.

As further evidence that the differential penetration of oxygen in sea water in receptacles of different capacities does not account for the increased bacterial activity in small volumes, sea water having different initial oxygen contents was stored. Twenty liters of freshly collected sea water was deoxygenated by holding it for two hours at a pressure reduced to less than 1 cm. of mercury with a High-Vac pump during which time the water was agitated. Then pure nitrogen was used to

force the water into 120-cc. bottles which were immediately stoppered and sealed. Under these conditions sea water containing only 0.59 cc. oxygen per liter was obtained. Some of this deoxygenated water was mixed with oxygen-saturated sea water to give a series having an initial oxygen content of 3.08 cc. per liter. As a control some of the deoxygenated water was saturated with oxygen by shaking it with air. It then contained 5.42 cc. of oxygen per liter. The tightly stoppered bottles were submerged in a water bath at 16° C. After different periods of incubation duplicate bottles were analyzed for bacteria and oxygen content. Table V presents the findings.

There was considerable bacterial activity in the deoxygenated water which contained only 0.59 cc. oxygen per liter as manifested by the rapid increase in the bacterial population and the depletion of the oxygen. As

TABLE V

Bacterial and oxygen content of sea water initially containing different concentrations of dissolved oxygen after storage at 16° C. in 120-cc. bottles.

Storage period	Oxygen-saturated water		Partly saturated water		Deoxygenated water	
	Bacteria per cc.	Oxygen in cc. per liter	Bacteria per cc.	Oxygen in cc. per liter	Bacteria per cc.	Oxygen in cc. per liter
Start	1,190	5.42	1,230	3.08	1,240	0.59
3 days	264,000	5.12	224,000	2.91	216,000	0.42
5 days	1,458,000	4.68	1,170,000	2.48	660,000	0.22
7 days	792,000	4.35	1,226,000	2.26	683,000	0.00
10 days	116,000	3.82	469,000	1.65	152,000	0.00
18 days	77,000	2.90	17,000	1.19	28,000	0.00

a matter of fact, the multiplication rate in the deoxygenated water was only slightly less than in the oxygen-saturated water until the oxygen was depleted. Likewise it will be observed that the amount of oxygen consumed during the first seven days was virtually the same regardless of the initial oxygen content of the water. Bacterial multiplication in sea water having a low oxygen tension was expected because most marine bacteria are microærophiles and many of them are facultative anaerobes. However, the rate of multiplication and oxygen-consumption in the water of low oxygen tension proved to be surprisingly rapid and these results are reported only after having been repeated.

Finally, similar volumes of water were placed in receptacles of different shapes so that different surface areas were exposed to the air. For example, 500 cc. quantities of water were placed in liter Roux flasks, in 500 cc. graduate cylinders and in glass tubes 2.1 cm. in diameter by

140 cm. long. When a Roux flask was placed on its side, 220 sq. cm. of water surface was exposed to air. An identical volume of water in a Roux flask stood on end exposed only 40 sq. cm. of water surface to air. In the 500-cc. cylinder 16 sq. cm. was exposed to air and in the 2.1 cm. tube only 3.2 sq. cm. of the water surface was exposed to air. Although it is recognized that sampling difficulties introduced certain errors, the bacterial populations were found to be in no way related to the area of water surface exposed to air. However, a relationship was noted between the area of glass surface exposed to water and the bacterial populations in stored sea water.

Effect of Solid Surface

It is well known that the smaller the capacity of similarly shaped receptacles the greater is the surface area per unit volume. This may be illustrated by the data in Table VI which gives the dimensions of the

Table VI

*Volume of sea water stored in different receptacles, area of solid surface in contact with the water, ratio of volume in cc. to area in sq. cm. and the average * maximum bacterial populations per cc. after storage at 16° C.*

Volume of sea water in cc.	Solid surface in sq. cm.	Ratio of cc. : sq. cm.	Average maximum * bacteria per cc.
14	37	1 : 2.64	1,863,000
120	148	1 : 1.23	1,070,000
1,225	640	1 : 0.52	553,000
13,220	3174	1 : 0.24	261,000

* Average of three different experiments.

receptacles which have been in use in the foregoing experiments. The average maximum number of bacteria which have been found in these receptacles in three different experiments is also given. It will be observed that the densest bacterial populations appear in the bottles of water which offer the largest area of glass surface per unit volume of water. As reported above, this was likewise found to be true when the volume was kept constant (500 cc.) and the shape of the receptacles varied to give different glass surface areas.

Next receptacles were selected so that their volumes varied greatly but the ratios of glass surface to volume remained practically constant. This was accomplished by using different lengths of glass tubing 2.1 cm. in diameter, one end of which was sealed and the other end stoppered. From 28 to 925 cc. of sea water was stored in such cylinders and within

the limits of experimental error the growth curves ran practically parallel. Table VII summarizes typical bacteriological findings in experiments in which receptacles of assorted capacities and shapes giving a wide range of ratios of volume to solid surface were used for storing sea water. Prior to being used all the glassware was aged in sea water, cleaned with hot sulfuric acid dichromate solution, rinsed with distilled water and sterilized. While the expression of the relationship of glass surface to bacterial populations defies exact mathematical expression, it will be noted that: in general, the larger the ratio of volume to surface

TABLE VII

Volume of sea water stored in different receptacles, area of solid surface in contact with the water, ratio of volume in cc. to area in sq. cm. and the average maximum bacterial population following storage at 16° C.

Receptacle	Volume of sea water	Area of solid surface	Ratio of cc. : sq. cm.	Bacteria per cc.
1.0 cc. pipette	1.0 cc.	9.9 sq. cm.	1 : 9.9	2,480,000
Blake bottle on side	10.0 cc.	98.7 sq. cm.	1 : 9.9	3,120,000
1.8 cm. test tube	1.0 cc.	4.1 sq. cm.	1 : 4.1	1,895,000
2.5 cm. test tube	10.0 cc.	20.3 sq. cm.	1 : 2.0	855,000
2.1 cm. glass tube, 8 cm. long	28.0 cc.	50.4 sq. cm.	1 : 1.8	970,000
2.1 cm. glass tube, 140 cm. long	550.0 cc.	925.0 sq. cm.	1 : 1.7	720,000
Nest of glass tubes, 140 cm. long *	410.0 cc.	2,556.0 sq. cm.	1 : 6.2	1,470,000
Liter bottle	1,225.0 cc.	640.0 sq. cm.	1 : 0.5	490,000
Liter bottle with 4,100 3 mm. glass beads	1,067.0 cc.	1,690.0 sq. cm.	1 : 1.7	642,000
Liter bottle with 1,350 cm. 3 mm. glass rods	1,099.0 cc.	1,840.0 sq. cm.	1 : 1.7	598,000

* The nest of glass tubes consisted of a piece of 2.1 cm. glass tubing 140 cm. long in which was placed three glass cylinders nearly as long and 1.6, 1.0 and 0.6 cm. in diameter respectively.

area, the larger the bacterial populations regardless of the total volume of stored sea water.

In the liter bottles in which the ratios of volume to solid surface were increased by placing a shallow layer of 3-mm. glass beads or glass rods in them, the plate counts were higher than in ordinary liter bottles but the increases were not proportional to the additional surface area nor were they as high as in other receptacles having the same ratios (see Table VII). This is considered as evidence that another factor besides solid surface is involved which influences the activity of bacteria in stored sea water. Further proof was obtained by covering the bottoms of liter bottles with enough glass wool or fine silica sand to give ratios exceeding 1 : 1,000. Following the storage of sea water under these conditions, the

plate counts were only slightly higher than in ordinary liter bottles having ratios of only 1:0.5 and about the same as in liter bottles containing enough 3-mm. glass beads to give ratios of 1:1.7. The explanation is believed to be found in the proximity of the water to a solid surface or in the distribution of the solid surface throughout the water. For example, in a liter bottle whose diameter is 10.6 cm., part of the water is at least 5 cm. away from the nearest solid surface whereas in a 2.1 cm. tube, the greatest distance is only about 1 cm. and within the interstitial water between 3-mm. glass beads the greatest distance of any particles of water to the nearest solid surface is only a fraction of a millimeter. Thus while a layer of glass beads or sand in the bottom of the bottle increases the surface per unit volume, the additional solid surface affects only that part of the water which is in the proximity of the glass beads or sand.

Sea water stored within the interstices of small inert particles such as sand or glass beads supported larger bacterial populations than it did under any other condition of storage. Fine silica sand was treated with hot sulfuric acid dichromate cleaning solution and washed with distilled water until the reaction was neutral. The clean sand was then dried in an oven after which 25-gram portions were weighed into 100-cc. Pyrex bottles. These bottles containing sand were heated to 240° C. for 6 hours. When cool, the sand was moistened with 10 cc. of sea water which was just enough to barely cover the sand. After varying periods of storage the sea water and sand mixture was washed into a 900-cc. water blank with 90 cc. of sterile sea water. Plating dilutions of this revealed the presence of from six to twelve million bacteria per cc., whereas less than a million per cc. appeared in identical sea water when 10 cc. was stored in similar bottles without sand. Between four and six million bacteria per cc. were demonstrated in the sea water with which the small glass beads in a 100-cc. bottle were covered. Controls consisting of clean sand or glass beads covered with distilled water and inoculated with a mixed microflora showed no appreciable bacterial multiplication, thereby indicating that neither the sand nor the beads supplied utilizable nutrients. The high counts which are obtained when sea water is stored in sand or glass beads are attributed to the proximity of the water to solid surfaces. There may be even more bacterial multiplication under these conditions than is indicated by the plate counts because it is improbable that all of the viable bacteria are recovered from the sand or beads.

Early in this work it was recognized that marine bacteria have a tendency to adhere to solid surfaces. Consequently in all of the experiments the water was vigorously shaken prior to sampling in order to recover

as many bacteria as possible. Usually the bottles were only partly filled with water to permit thorough mixing except in the experiments dealing with oxygen consumption in stored sea water. The 1- to 10-cc. volumes of stored sea water were poured directly into the dilution water and the receptacle in which the water had been stored was rinsed out several times. Receptacles which were filled to capacity were partly emptied into sterile vessels to permit shaking. The sea water stored in long glass tubes was poured out and in several times to wash the bacteria from the glass surfaces to which they adhere.

In order to show quantitatively the tendency of bacteria to adhere to the glass, 100 cc. of sea water was stored in 120-cc. bottles, two of which were analyzed daily. First, all of the water was transferred to another vessel without any shaking and this was appropriately diluted and plated.

TABLE VIII

Number of bacteria per cc. in 100 cc. of sea water poured from bottles in which it had been stored without shaking and the number per cc. in the first and second 100 cc. of sea water with which the original bottle was rinsed.

Storage period	Number of bacteria per cc.		
	Original water	First rinse water	Second rinse water
Start	2,800	0	0
2 days	65,000	48,000	2,600
3 days	108,000	67,000	3,900
4 days	220,000	90,000	10,600
5 days	278,000	122,000	6,450
6 days	270,000	136,000	10,200
10 days	10,000	120,000	9,600

Then the original bottle in which the sea water had been stored was thoroughly rinsed with 100 cc. of sterile sea water and this was poured off and plated. The rinsing process was repeated with a second 100 cc. of sterile water which was plated. The resulting plate counts are given in Table VIII. The data show that on an average nearly half of the bacteria in the bottles of stored sea water were stuck firmly enough to the walls of the bottle to resist being detached by pouring off the water without shaking.

The foregoing procedure fails to demonstrate all of the bacteria which are attached to the glass because many are not washed off by any amount of rinsing in water. After being rinsed with several 100-cc. portions of water, 20 cc. of melted nutrient agar cooled to 45° C. was poured into the original bottle in which the sea water had been stored and the agar was permitted to solidify on the inside periphery of the

bottle Esmarch-tube fashion. Following incubation too many colonies developed to permit their enumeration.

Direct microscopic examination (Henrici, 1933) of slides which were suspended in liter bottles of stored sea water for 24 hours revealed the presence of over 100,000 bacteria per sq. cm. If bacteria were equally abundant on all of the glass surface within the bottle, it is calculated that for each cc. of water there was the equivalent of 84,000 bacteria attached to the glass while the water itself contained only 31,000 bacteria per cc. including those which could be dislodged by shaking vigorously the bottle of water. The proponderance of attached bacteria increases progressively during storage until they become too thick to enumerate with precision. It is recognized that all of the bacteria observed microscopically may not be alive and also that plate counts do not demonstrate all of the viable bacteria in the water. Therefore the data obtained by the two procedures are not directly comparable. However, the data conclusively demonstrate that large numbers of bacteria in stored sea water attach to glass surfaces. The development of micro-colonies on the slides immersed in sea water indicates that many of the attached bacteria are alive and multiplying. The respiration of these attached bacteria is believed to account for the oxygen consumption which continues to occur in stored sea water after the bacterial population of the water has dropped to a low level (see Fig. 2).

In these experiments the bacteria were not fixed to the slides by heat or otherwise. The criterion of periphytic bacteria is their ability to attach themselves so tenaciously to the slide that they are not dislodged by washing in water and the other staining procedures. According to ZoBell and Allen (1933) appreciable numbers of bacteria attach themselves to glass slides within 2 to 4 hours after being submerged in sea water. They (1935) found as many as 720,000 bacteria per sq. cm. after the slides had been suspended in the sea for 24 hours. Nearly a billion bacteria per sq. cm. have been observed on glass slides submerged in the sea for a week.

Discussion

The maximum bacterial population which sea water filtered free of particulate matter can support is probably between ten and one hundred million per cc. (including estimated periphytes). Bacterial multiplication in stored sea water is undoubtedly limited by organic matter because over a billion bacteria per cc. have been found in sea water enriched with 100 mgm. of peptone per liter. However, when enriched there is no apparent difference in the activity of bacteria in different volumes of

sea water. The fact that the volume effects occur only in very dilute nutrient solutions may explain why exhaustive growth curve studies of other investigators have not shown volume of the media or solid surfaces to be a factor which influences bacterial multiplication. Most growth curve studies on saprophytes have been conducted in media containing at least 1,000 mgm. of organic matter per liter (Rahn, 1932). Using different carbon compounds Stephenson (1930) shows that 10 to 100 mgm. per liter is the minimum concentration in which *Bact. coli* and certain other bacteria can grow. It will be recalled that sea water contains less than 10 mgm. of organic matter per liter.

Multiplication and oxygen consumption are not the only bacterial activities in stored sea water which are influenced by volume or surface. It was found that the minimum concentration of organic matter which limits denitrification was considerably less in 10-cc. quantities of sea water than in 500-cc. quantities. This was not understood until the effect of solid surface on bacterial activities in dilute nutrient solutions was studied. Several Erlenmeyer flasks varying in size from 25 to 6,000 cc. were filled with raw sea water enriched with 10 mgm. peptone and 1.0 mgm. KNO_2 per liter. It was found that nitrite was depleted from the smallest flasks first and from the largest flasks last or not at all. When attempts were made to accelerate the reaction by adding 100 mgm. or more of peptone per liter, there was no difference between small and large receptacles in the rate of nitrite reduction. Changes in the pH and ammonia content likewise occur more rapidly in sea water stored in small receptacles than in identical sea water stored in large receptacles.

The volume effects are attributed to the favorable influence of solid surfaces and to the proximity of these surfaces to the water particles. Solid surfaces are believed to favor bacterial activity in dilute nutrient solutions in several ways. First, they provide a resting place for periphytes. In discussing the advantages of semi-solid media, Hitchens (1921) suggests that bacteria may need objects upon which to cling while growing. It is difficult to estimate what proportion of marine bacteria are periphytes but ZoBell and Allen (1933) have demonstrated that many species have periphytic tendencies and that at least some of them are obligate periphytes. This is in agreement with field observations. Lloyd (1930) calls attention to the fact that the saprophytic bacteria of the sea are not planktonic but are attached to suspended particles. Waksman and co-workers (1933) find a definite parallelism between the bacteria and plankton in sea water and they conclude that "bacteria exist only to a very limited extent in the free water of the sea, but are largely attached to the plankton organisms." This conclusion is cor-

roborated by the literature cited by Benecke (1933) dealing with the distribution of bacteria in the sea.

Secondly, solid surfaces favor bacterial activity in dilute nutrient solutions because the nutrients may become concentrated in a film upon the solid surface due to adsorption or other kinds of physical attraction. Studies on the micro-organic films which form on glass slides soon after being submerged in sea water give some evidence of such a concentration. The accumulation of a film of organic matter upon submerged slides can be demonstrated by differential stains as well as by micro-chemical analyses. This hypothesis is supported by the fact that the favorable effect of solid surfaces is most striking in dilute nutrient solutions and by the fact that a large percentage of the bacteria multiply on the glass surface although many of them are not firmly attached to it.

Third, the solid surface, the capsular-like material which constitutes the holdfast of periphytic bacteria and the adsorbed organic matter may help retain the exo-enzymes near the bacteria until they can digest and ingest the nutrients. The interstices at the tangent of the bacterial cell and the glass surface may serve as concentration foci which retard the diffusion of the enzymes and the digestion end-products away from the cell. The inhibiting effect of continuous gentle agitation on the multiplication of bacteria in stored sea water is attributed to an acceleration in the diffusion of either the exo-enzymes or the digestion end-products away from the bacterial cells. It is generally recognized that bacterial adsorption to particles exerts a favorable influence on bacterial enzymatic activity.

Besides being of interest to students of bacterial physiology, this solid surface phenomenon is believed to be an important factor in explaining the vertical distribution of bacteria in the sea. In the ocean, bacteria are most abundant in the zone 10 to 50 meters below the surface where there are usually a few hundred or less bacteria per cc. Bacteria are only rarely encountered at depths exceeding 200 meters except on the sea floor where the bottom slime may contain millions of bacteria per cc. regardless of the depth of the overlying water. However, water collected from depths exceeding 200 meters contains sufficient nutrients to give rise to ten to a hundred million bacteria per cc. when the water is stored in small volumes. Why then is this deep water so sparsely populated? As ZoBell (1934) has shown, it cannot be attributed to the prevailing low temperature or high hydrostatic pressure. In view of the foregoing experimental observations, it is believed that the dearth of particulate matter to serve as solid surfaces is a factor which restricts the activity of bacteria in deep water.

Summary

The storage of sea water is accompanied by a great increase in the number of bacteria and a decrease in bacterial species.

Appreciably more bacteria per cc. appear in small volumes of sea water than in large volumes of identical water.

Although the oxygen content of sea water is a factor which influences the activity of bacteria, it does not account for the denser bacterial populations which appear in the smaller volumes.

The favorable influence of small volumes is attributed to the contact of the water with the larger solid surface area in the small receptacles. Between ten and a hundred million bacteria per cc. have been demonstrated in sea water stored in sand which presents an enormous surface area whereas less than three hundred thousand bacteria per cc. appear in similar water stored in 10-liter bottles presenting relatively little surface area.

The beneficial effect of small volumes and their large surface areas occurs only in dilute nutrient solutions. Upon the addition of 10 to 100 mgm. of organic matter to sea water, the volume effects disappear.

Solid surfaces may serve as a resting place for periphytes, they may help concentrate the nutrients by adsorption or otherwise, or they may favor bacterial enzymatic activity and the absorption of metabolites.

Appreciation is here expressed for the criticisms and suggestions of Drs. Blodwen Lloyd, Austin Phelps, and Roger Revelle. Acknowledgment is also made to Mr. Hiomi Nakamura who made the oxygen determinations and to Mr. Carl I. Johnson for technical assistance.

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THE UTILIZATION OF SOLUTES BY MOSQUITO LARVÆ

WILLIAM TRAGER

(From the Department of Animal and Plant Pathology of the Rockefeller Institute for Medical Research, Princeton, N. J.)

Introduction

One of the important factors conditioning the suitability of a habitat for the development of mosquito larvæ is the available food supply. Chiefly for this reason, a number of investigators (see Hinman, 1930, for a summary of the literature) have studied the food requirements of mosquito larvæ. Since these insects usually ingest large amounts of particulate matter, it has been commonly concluded that this material is the principal, if not the only, source of food. However, in 1907, Pütter had shown that the chief food source for some aquatic organisms consists of organic colloids and solutes, and Matheson and Hinman (1930) suggested that such was probably the case with mosquito larvæ. Hinman (1930) presented evidence in support of this suggestion. He was able to rear small numbers of sterile *Ædes ægypti* in water very rich in organic matter that had been filtered through a Berkefeld N filter. Later (Hinman, 1932) he found that anopheline larvæ, in Berkefeld filtrates of the water of their natural habitat, could survive about 11 days and that some reached the second instar. If such water was subjected to dialysis against distilled water, and the dialysate sterilized by Berkefeld filtration, a medium was obtained containing only the solutes of the original water. In such a medium, sterile anopheline larvæ died within 3 days without reaching the second instar. Shipitsina (1930) obtained results similar to those of Hinman. In Berkefeld filtered water, anopheline larvæ made a slight growth, some reaching the second instar. She concluded that while organic colloids play some part in the nutrition of the larvæ, by far the larger rôle must be attributed to particulate matter.

Neither Hinman nor Shipitsina obtained any conclusive data concerning the ability of mosquito larvæ to utilize substances in true solution. The work upon this problem to be reported in this paper was made possible by the development (Trager, 1935*a*, *b*) of a reliable method of rearing mosquito larvæ free from microörganisms. Three different lines of evidence were obtained all supporting the view that mosquito

larvæ can utilize solutes. First it was found that a growth factor required by the larvæ and present in Lilly liver extract No. 343 is water-soluble. Then a study of the effects of several substances of known chemical composition and known to exist in water in true solution showed that one of them, calcium chloride, is a substance essential for the normal development of the larvæ. Finally, it was possible to rear mosquito larvæ to the fourth instar in media ultrafiltered through collodion.

Methods

Recently laid eggs of the yellow fever mosquito, *Ædes ægypti* (a stock colony of which was maintained), were freed from microörganisms by a 15-minute immersion in a fluid recommended by White (1931) and consisting of mercuric chloride 0.25 gram, sodium chloride 6.5 grams, hydrochloric acid 1.25 cc., ethyl alcohol 250 cc., and distilled water 750 cc. The eggs were handled in small glass "boats" (MacGregor, 1929) made from coverslips, were rinsed in sterile tap water, and inoculated into tubes containing an autoclaved 0.5 per cent solution of Lilly liver extract No. 343. The bacteria-free larvæ which subsequently hatched in this medium were washed in sterile distilled water and counted numbers of them were inoculated into the experimental tubes. For most of the work 18×160 mm. test tubes holding 6 cc. of medium were used. Each tube received three larvæ. The tubes were incubated at $28 \pm 1°$ C. and were observed daily or every other day, the number of larvæ in each stage of development in each tube being noted. Figure 1 is a photograph of a typical culture tube in which one of the three larvæ has already pupated while the other two are in the fourth instar. Aseptic precautions were observed throughout, all the tubes were tested for sterility, and contaminated ones were discarded.

Evidence that the Growth Factor (*A*) Exists as a Solute

The first evidence that the larvæ could utilize substances in true solution was obtained in the course of the study of the properties of the accessory growth factor present in liver extract (factor *A*). In the presence of whole killed yeast and at concentrations of Lilly liver extract No. 343 below 0.5 per cent, the growth of *A. ægypti* larvæ is directly proportional to the concentration of liver extract and hence of factor *A* (Trager, 1936). There can thus be no doubt of the utilization of factor *A* by the larvæ. When a solution of liver extract 343 was dialyzed 24 hours in a collodion bag against an equal volume of distilled water, the concentration of *A* was as great in the outer as in the inner liquid. The factor thus dialyzes readily and must be present in true solution.

The Effects of Certain Solutes of Known Chemical Composition

Tubes were prepared each containing about 30–40 mg. powdered Harris vitamin B-free casein. They were sterilized by dry heat and to each tube was added the desired liquid medium, previously sterilized by autoclaving or by Berkefeld filtration. In tubes with casein and distilled water, first instar larvæ died within 2 to 3 days. The presence

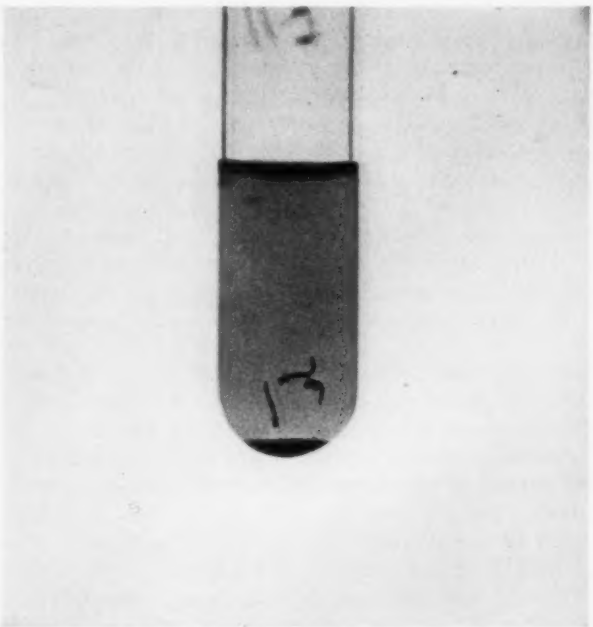

Fig. 1. Photograph of a tube containing casein, yeast extract, and liver extract. One of the three larvæ has pupated while the other two are in the fourth instar. Note the casein at the bottom of the tube. Photographed by J. A. Carlile.

of 0.1 M glucose or sucrose did not prolong their life. However, with 0.1 M maltose several larvæ survived about a week. In the presence of a salt mixture containing NaCl, $CaCl_2$, $NaH_2PO_4 \cdot H_2O$ and K_2HPO_4 in a total concentration of 0.05 M, several larvæ survived over a week, while one reached the second instar and lived for 22 days. This would indicate an effect of the soluble salts on the longevity of small larvæ which were unable to grow because of the absence of accessory growth factors.

Experiments were then performed to test the effect of solutions of salts and sugars on the growth of larvæ in the presence of accessory growth factors. It was previously shown (Trager, 1935*b*) that *A. ægypti* larvæ could be reared to maturity on a diet of casein, yeast extract (0.3 per cent yeast vitamin Harris) and liver extract 343. It was later found (Trager, 1936) that yeast extract contains the accessory growth factor present in liver extract (factor *A*) as well as a second accessory factor. It was accordingly possible to rear larvæ to maturity on a diet of sterile casein and autoclaved yeast extract. The minimum concentration of yeast vitamin Harris needed to supply enough factor *A* was 0.8 per cent. In such a medium, half of the larvæ inoculated would usually reach the adult stage within about 3 weeks.

This medium, consisting of casein and 0.8 per cent yeast vitamin Harris, was adopted as a basic medium and into it were incorporated various salts and sugars. Glucose, sucrose, and maltose in concentrations of 0.15, 0.10, and 0.05 M gave no better growth than that in the basic medium alone. The same was true of NaCl solutions in concentrations of 0.075, 0.050, and 0.025 M, as well as of various NaCl–KCl and NaCl–$MgSO_4$ mixtures. In all these media, on the sixteenth day, none of the larvæ had pupated and only about half had reached the fourth instar. But in the presence of both NaCl and $CaCl_2$ a striking acceleration of growth was noted. In a medium containing 0.04 M NaCl and 0.01 M $CaCl_2$ all of the larvæ reached the fourth instar in an average time of 8.5 days, two-thirds pupated in an average time of 12.5 days and gave normal adults [1] 2 days later. In a medium containing 0.045 M NaCl and 0.005 M $CaCl_2$ all the larvæ reached the fourth instar in an average time of 6 days, all pupated in an average time of 10.5 days and gave normal adults [1] 2 days later. The total time of development thus averaged 12.5 days, as compared to 9 days on a rich nutrient medium of whole dead yeast and liver extract. When other salts, or sugars, were substituted for part of the NaCl and $CaCl_2$ combination, growth was somewhat less than in the medium with 0.045 M NaCl and 0.005 M $CaCl_2$.

In another experiment a comparison was made of growth in the same basic medium in the absence of added salts and in the presence of concentrations of $CaCl_2$ ranging from 0.05 to 0.005 M. Growth in the presence of $CaCl_2$ was much more rapid than in its absence and was as good as in the presence of both NaCl and $CaCl_2$. These results show that $CaCl_2$, known to be in true solution in water, is an additional factor conditioning the growth of *A. ægypti* larvæ.

[1] These adults were, however, smaller than those obtained in richer nutrient media.

Growth in Nutrient Media Containing Only Solutes

In previous work (1935*b*) it was found that *A. ægypti* larvæ grew quite normally in media consisting of yeast autolysate and liver extract, or yeast extract, casein digest and liver extract. Such media, sterilized by autoclaving, contained few particles but undoubtedly contained much material in colloidal suspension. From these results it is apparent that the insects can develop to the adult stage on a diet supplied either wholly in the form of colloids or partly as colloids and partly in true solution.

Several attempts were then made to rear the mosquito larvæ in media containing nothing but solutes. It had been found that fair growth could be obtained in autoclaved 2.5 per cent solutions of Vegex, a commercial yeast extract. These solutions after autoclaving contained some sediment. Three collodion bags were prepared each containing a fresh 5 per cent solution of Vegex and surrounded by an equal volume of distilled water. Both inner and outer liquids were covered with toluene and the dialysis was allowed to proceed 2 days at room temperature. The inner and outer liquids were then separated from the toluene, tubed, and autoclaved. No visible sediment formed in the dialysate after autoclaving. Each of two tubes of each solution was inoculated with three larvæ. The results follow.

Preparation 1.	Outer liquid.	One reached 3rd in 13 days, 4th in 22 days, none pupated.
	Inner liquid.	Three reached 3rd in 8.5 days, 2 reached 4th in 24 days, none pupated.
Preparation 2.	Outer liquid.	Two reached 3rd in 8 days, none reached 4th.
	Inner liquid.	Five reached 3rd in 10.5 days, 3 reached 4th in 23 days, 1 pupated in 35 days.
Preparation 3.	Outer liquid.	One reached 3rd in 10 days, 4th in 32 days, none pupated.
	Inner liquid.	Five reached 3rd in 9 days, 4 reached 4th in 29.5 days, none pupated.
Untreated 2.5 per cent Vegex.		Four reached 3rd in 6 days, 3 reached 4th in 12.5 days, 3 pupated in 19.5 days.

It is evident that in all three dialysates there was significant growth of the larvæ. It should be remarked that *all* the larvæ in these solutions reached the second instar and that the growth of some to the third and

fourth instar did not take place as a result of cannibalism. In all cases there was more growth in the inner liquid than in the outer or dialysate. But the growth in the inner liquid was not as good as in untreated 2.5 per cent Vegex. Probably considerable amounts of nutrient material were adsorbed by the collodion membranes, which acquired a brown color.

A solution was prepared containing 1 per cent liver extract 343, 1 per cent Fairchild's peptone, and 2 per cent Difco yeast extract. Part of this was sterilized by filtration through a Berkefeld N filter. Another part was filtered under pressure through a disc-shaped collodion membrane held in a Seitz filter. The ultrafiltrate so obtained was sterilized by Berkefeld filtration. Each of the two liquids was tested alone and with casein, 6 larvæ being used for each test. The results were as follows.

Not ultrafiltered.	Six reached 3rd in 6.5 days, 2 reached 4th in 13 days, none pupated.
Same with casein.	Six reached 3rd in 4.5 days, 1 reached 4th in 12 days, none pupated.
Ultrafiltrate.	Three reached 3rd in 8.5 days, 2 reached 4th in 25 days, none pupated.
Same with casein.	Two reached 3rd in 9 days, 1 reached 4th in 13 days, none pupated.

Even in the solution filtered only through a Berkefeld and in the presence of casein, growth was not as good as in similar solutions sterilized by autoclaving. The Berkefeld candle probably adsorbed part of the required nutrients. Growth was not markedly affected by ultrafiltration or by the presence of solid protein in the form of casein. In another similar experiment a solution was prepared containing a fuller's earth extract (as a source of salts), 0.5 per cent liver extract 343, 1.2 per cent Vegex, and 2 per cent Fairchild's peptone. This was filtered under pressure through a collodion disc and was sterilized by Berkefeld filtration. Of 6 larvæ inoculated into this medium, 5 reached the third instar in 7.5 days and 2 reached the fourth in 14 days, but none pupated. The fourth instar larvæ in this, as in the preceding experiments, never attained the size of normal full-grown individuals.

Discussion

The effect of calcium chloride on larval growth is, quite apart from its bearing on the utilization of solutes, of sufficient interest to merit some discussion. A certain amount of this salt is necessary for the normal development of *Ædes ægypti* larvæ. The effect is not an osmotic one. Sugars or other salts in equivalent concentrations do not show it.

Previous work on the effects of salts on mosquito larvæ has been concerned mainly with the resistance of various species to high salt concentrations. Wigglesworth (1933*c*) found that *A. ægypti* larvæ were killed by 1.1 per cent sodium chloride but, by very gradually evaporating the solution, could be made resistant to this concentration and to artificial sea water of a concentration osmotically equivalent to 1.75 per cent sodium chloride. He called attention to the fact that the larvæ can resist higher osmotic pressures in balanced salt solutions than in solutions of a single salt, and concluded that death of the larvæ in both cases was due, not to osmotic effects, but to the presence of directly toxic concentrations of certain ions. The possible stimulating effect of low concentrations of salts has received little attention. Roubaud, Colas-Belcour, and Treillard (1935), however, noted that while higher salt concentrations (in the form of sea water) were toxic to larvæ of three races of *Anopheles maculipennis*, lower concentrations, ranging from one-fifth to one-twentieth the strength of sea water, were distinctly favorable and resulted in better growth than that obtained in distilled water. They state that this effect may be due to a stimulation by the salts on bacterial growth or to the physico-chemical state of the water. In the light of my own results, it seems probable that the favorable effect of low sea water concentrations observed by these workers was the result of the specific action of essential ions.

It is, of course, well known that electrolytes have remarkable effects on a wide variety of animals and plants. One need only mention the pioneer work of Ringer (1880–82), Loeb (1910), and Osterhout (1922). Calcium ions seem to be of especial importance in many biological phenomena (see Bayliss, 1915) such as the coagulation of milk and blood, the beat of the heart, the transmission of nerve impulses, etc. Recently Warén (1933) has shown that calcium is essential for cell division in the plant *Micrasterias*. It is, therefore, not surprising that calcium should be a growth requirement of mosquito larvæ. Whether the calcium is essential for its own sake, or because of its antagonism toward some other ion, cannot be said at present.

The effects on larval growth of calcium chloride, known to exist in water as a solute, and of factor *A*, shown to be also in true solution, demonstrate clearly the ability of the larvæ to utilize substances in solution. Moreover, in view of the results described with ultrafiltrates, there can be no doubt that *Ædes ægypti* larvæ can grow to the last larval instar at the expense of nutrients existing wholly in true solution. It seems probable that the subnormal growth in such media containing only solutes is due, not to the inability of the larvæ to utilize nutrients in such a state, but to the experimental difficulty of providing, in the state of

true solution, sufficiently high concentrations of some of the required substances. On the other hand, when solid food is available and the alimentary tract becomes packed with it, very high concentrations of soluble nutrients must be produced as a result of digestion. Such an explanation may very well apply to aquatic organisms other than mosquito larvæ. For example, the finding of Gellis and Clarke (1935) that *Daphnia magna* could grow in media containing colloids but not in media containing only solutes may be explained in this manner. The difference between nutrients existing in the environment as particles, colloids, or in true solution becomes a quantitative one. Whatever the initial state of the nutrient, it must be changed to a soluble form before it can be absorbed. When the nutrient exists as particles or even in colloidal form, the mosquito larvæ, or other aquatic organisms, are able to concentrate it by gathering up the particles. This they are not able to do with nutrients existing as solutes. These must enter the alimentary tract along with water and are present therein in the same concentration as in the surrounding fluid.

These considerations bring us to the problem of the method of ingestion of food by mosquito larvæ. The larval mouth brushes have been generally considered as an apparatus for gathering up fine particles. Shipitsina (1930) regarded them as filters capable of filtering out from the water even colloidal particles. She found that anopheline larvæ could filter out particles of colloidal silver with a diameter of 20 mμ but could not filter out the particles of soluble starch with a diameter of 5 mμ. Wigglesworth (1933*b*), using larvæ of *Ædes ægypti*, also concluded that very little fluid was ingested by mouth. He observed that the larva accumulates bits of food in its buccal cavity and does not swallow the food until a considerable bolus has accumulated. My own observations indicate that in particle-free media the brushes are probably of little use, but the swallowing movements of the larva enable it to ingest fluid.

Since larvæ can reach the fourth instar at the expense of nutrients in solution, these nutrients must somehow be absorbed by the larva. Howland (1930) has shown that dyes in solution in the external liquid can enter the alimentary tract of mosquito larvæ and be absorbed there. It seems most likely that soluble nutrients would follow the same path. However, Wigglesworth (1933*b*) found that, while the cuticle covering the body of mosquito larvæ is impermeable to water, water can and does regularly enter the body by way of the anal gills. The possibility of the entrance of nutrient solutes in this manner must be borne in mind, although Wigglesworth (1933*a*) has also shown that, while certain salts

can enter the anal gill cells from the external medium, they cannot pass from the cells into the hemolymph.

Summary

The larvæ of the yellow fever mosquito, *Ædes ægypti*, are able to utilize substances in true solution. They require for development at a normal rate a proper concentration of calcium chloride, known to exist in water as a solute. Their growth is likewise conditioned by an organic growth factor shown to be also a solute. Finally, the larvæ can develop at least as far as the fourth instar in media the nutrients of which exist entirely in true solution.

It gives me pleasure to acknowledge the helpful interest of Dr. R. W. Glaser.

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ENVIRONMENT AND SEX IN THE OVIPAROUS OYSTER OSTREA VIRGINICA

W. R. COE

(From the Osborn Zoölogical Laboratory, Yale University)

The Virginia oyster is a member of that group of oviparous pelecypods in which each individual, with relatively few exceptions, is seasonally functional as either male or female, with the possibility of reversal to the opposite sex during the interval between two breeding seasons (Needler, 1932; Coe, 1932*a*, 1934; Burkenroad, 1936). All species of larviparous oysters so far as known have a more or less regularly alternating rhythm of male and female phases.

In the northern part of its range the Virginia oyster appears to be mainly or wholly protandric, while in somewhat warmer waters, at least as far southward as Virginia, a considerable proportion of the young oysters function as females during their first breeding season, which occurs at the age of one year. The more favorable the conditions are for the rapid growth of the young oyster, the more likely is the initial male phase to be omitted or aborted. Hence those localities and seasons in which the yearling oysters reach the largest mean size usually have the largest proportion of females in the first breeding season (Coe, 1934).

From North Carolina southward young oysters of both sexes reach functional maturity when only ten to twelve weeks of age.

In the older age groups the two sexes occur in approximately equal proportions, with some tendency toward an excess of males at the second breeding season and an excess of females in groups that are more than five years of age. It is unknown whether every individual changes its sex during even a long lifetime nor is it known whether more than a single change ever occurs.

The histological basis for these changes of sex depends upon the essential bisexuality of the gonads. In the young oyster before sexual maturity the gonad consists of a cortical layer of ovogonia surrounding a group of spermatogenic cells. In the typical case of protandry the spermatogonia proliferate rapidly, soon giving the gonad its dominantly male characteristics. Some ovogonia or ovocytes can nearly always be

detected in the cortex, however. These, together with many sexually undifferentiated cells, remain as residual gonia after the functional male phase has been completed. The sexual phase for the ensuing year will then depend upon which of the two alternative types of cells are stimulated to renewed activity.

In the sexually immature young oyster which is to function as a female at its first breeding season, on the other hand, the proliferation of the spermatogonia in the primary bisexual gonad is soon checked and is succeeded by a differentiation and multiplication of ovogonia. The initial male phase is thus aborted, sometimes even after the appearance of a few spermatids.

In approximately one per cent of the yearling population neither type of sexuality is fully dominant, so that partial or complete hermaphroditism results. Self-fertilization is successful experimentally and presumably occurs occasionally in nature.

Seasonal protandry. Occasionally the gonad of the yearling hermaphrodite shows a typical ovarian structure except for groups of spermatogenic cells or ripe spermatozoa next the lumens of some of the follicles. In some of these cases the sperm mature and are discharged early in the breeding season, leaving the ovocytes and a few residual spermatozoa to be discharged later during the same summer, as is the case with the young of viviparous species (Coe, 1932*b*).

During the past few years evidence has been obtained which gives some indication as to the influence of environmental conditions in determining which of the two alternative types of cells in the primary bisexual gonad shall be stimulated to proliferation; that is to say, which of the alternative sexual phases shall be functional at the breeding season.

It has been previously shown (Coe, 1932*a*, 1934) that the mean size of the females in the yearling group from each locality considerably exceeds that of the males. In addition to the data previously presented, further evidence was obtained from the examination of the gonads of more than 3,000 individuals from the vicinity of New Haven, Connecticut, during their first breeding season, in 1935 and 1936.

Of 670 young oysters which had been collected in the harbor and transferred to deep water off the New Haven Breakwater there was a ratio of 7.66 females to each hundred males. The mean size of the females was 26.88 mm. as compared with 25.43 mm. for the males. Similar collections of 391 yearlings made at the same season from the natural beds at Milford, only about eight miles distant, gave a ratio of 24 females for each hundred males, the females at this locality having a mean length

of 30.52 mm. and the males 30.24 mm. Tables I and II indicate the sex ratios which were found at several localities along the coast in the breeding seasons of 1932–1935, the entries listing immature individuals representing samples taken early in the season. The size of the shell at date of collection will obviously depend upon the length and favorableness of the two growing seasons in the young oyster's lifetime, but the size at the end of winter, at which time the sexual condition for the following season is presumably already determined, likewise shows an average larger size for females than for males.

TABLE I

Ostrea virginica. Sex at first breeding season. *Im* = sexually immature early in breeding season; *F* = female; *H* = hermaphroditic; *M* = male.

Locality	No. *Im.*	No. *M.*	No. *H.*	No. *F.*	Total	Percentage *F.*
New Haven (1932)	17	389	4	13	423	3.07
Great South Bay, L. I.	21	197	0	7	225	3.11
New Haven (1933)	1	129	0	7	137	5.11
New Haven (1935)	105	521	4	40	670	5.97
Milford (1935)	2	312	2	75	391	18.89
W. Sayville, L. I., (float)	0	154	4	48	206	23.30
Beaufort, N. C.	0	43	0	21	64	32.81

TABLE II

Ostrea virginica. Correlation of sex ratio and size. Mean length at first breeding season. *F* = female; *M* = male.

Locality	No. *M.*	Mean	No. *F.*	Mean	Ratio *F* to 100 *M*
		mm.		mm.	
New Haven (1932)	389	31.28	13	38.54	3.34
Great South Bay, L. I.	197	Ca. 29.	7	Ca. 34.	3.55
New Haven (1933)	129	39.90	7	44.14	5.42
New Haven (1935)	521	25.43	40	26.88	7.66
Milford (1935)	312	30.24	75	30.52	24.04
W. Sayville, L. I., (float)	154	46.33	48	59.33	31.17
Beaufort, N. C.	43	53.5	21	60.5	48.84

These results are in complete harmony with the evidence reported previously for other years and for other localities (Coe, 1932*a*, 1934). The data given in Tables I and II indicate that environmental conditions in the same locality may vary considerably from year to year, if it be

assumed that samples of several hundred individuals are truly representative of the entire population.

Date of setting and length of growing season. Since the sex ratio of the yearlings is evidently correlated with the size at the end of winter as well as with the size at the first breeding season, it seems probable that the length as well as the favorableness of the growing season preceding the first winter may influence the proportion of females. Years in which much of the set occurs in early August presumably show a higher ratio of females among the yearlings of the following year than is the case when most of the set takes place in September, providing the environmental conditions are similar.

The time of sexual maturity of the yearlings also shows much variation in different years and at various localities (Table I). In the northern portion of the species' range a large proportion of the young remain sexually immature until their second year (Needler, 1932) but south of Cape Cod most of them become functional at some time during the summer at the end of their first year. It was previously reported (Coe, 1932*a*, 1934) that in 1932 most of the yearlings in the Woods Hole area postponed spawning until their second summer, but in more recent years nearly all have become functional before the end of the first breeding season.

Change of sex ratio during breeding season. The progressively increasing ratio of mature females in the yearling population as the season advances is shown by recent collections from Milford, Connecticut, obtained through the courtesy of Dr. Victor L. Loosanoff of the Bureau of Fisheries station at Milford. At the beginning of the breeding season, July 3, 1936, a sample containing 911 yearlings was composed of 77 sexually immature individuals, 780 ripe males, 10 hermaphrodites and 44 females. A second sample, taken August 13, at the height of the breeding season, consisted of 7 individuals that were still sexually immature, 410 males, 4 hermaphrodites and 58 females. A third sample near the end of the breeding season contained 92 males and 21 females, besides 176 individuals which had almost completely spawned out. For each hundred males there were 5.64 functional females in the first sample, 14.15 in the second and 22.83 in the third sample. The increased ratio of females resulted in part from the maturity of females which had not become functional at the beginning of the season and in part from individuals which would have been classed as hermaphroditic males earlier but have later transformed to females by the maturation of the spermatogenic cells and discharge of the resulting spermatozoa.

The breeding season occurs earlier on the oyster beds in shallow, warmer water but varies with the temperature from year to year. From one bed in Milford in July, 1935, the female ratio in a sample of 312 yearlings was 24.04, while from another bed in the same month of 1934 it was only 7.50. On July 28, 1936, 373 yearlings from New Haven harbor showed a female ratio of 12.54, as compared with 5.42 in 1933 and 7.66 in 1935.

The distinctly larger mean size of the females during the first breeding season is well illustrated by random samples consisting of 202 individuals taken from the culture floats at West Sayville. Of those having a shell length of more than 50 mm. there were 49 males and 42 females, while the group with a length of less than 50 mm. contained 105 males and only 6 females. If the selection had been made either by volume or by weight the superiority of the females would have been even more evident.

Tables I and II offer substantial evidence that there is a rather close correlation between the mean size of the individuals in the yearling population and the percentage of functional females, or the ratio of females to males. The mean size of the females in all cases exceeds that of the males and with few exceptions those years and those localities in which the metabolic conditions are most favorable, as indicated by the size attained at the end of the first year, have the largest proportions of females.

With this additional evidence it now seems fair to conclude that favorable metabolic conditions increase not only the mean size of the individuals but also the proportion of females in the yearling population.

Three assumptions may be suggested in explanation of this conclusion: (*a*) that inherent sex factors are associated with metabolic factors that lead to the more rapid growth of those individuals that are to become females; (*b*) that the more favorable conditions for growth stimulate the proliferation and growth of the ovogonia in the young bisexual gonad or inhibit the proliferation of the spermatogonia; (*c*) that there is in the immature oviparous oysters an inherent tendency toward an alternation of sexuality such as is usual in the viviparous species (Orton, 1926–27; Coe, 1932*b*).

The first two assumptions have been discussed in a previous paper (Coe, 1934). The last assumption would imply that those individuals that are to function as females in their first breeding season, when one year of age, may have already passed through an aborted protandric phase in the late autumn of the year of hatching, when only about four months old. There is some evidence that this assumption is justified,

for it has been observed that the proliferation of spermatogonia and the incipient stages of spermatogenesis are most pronounced in the largest of the young individuals. It seems quite possible that these are the same individuals as those which function as females the following summer. The observed ratios in the various localities are in harmony with this supposition.

Protandry would thus dominate the sexuality of the oviparous species just as is the case in the viviparous forms (Coe, 1932*b*, 1934), the initial male phase being inaugurated, but aborted, in the autumn of the year of hatching for those individuals that function as females at the age of one year. If for any reason the protandric phase is delayed until the following spring, such individuals complete spermatogenesis and thus become functional males during the summer of the first breeding season. In those localities from Virginia northward which have the most favorable conditions for the inauguration of the aborted male phase in the autumn there will thus be the greatest proportion of females at the first breeding season.

It may be mentioned in conclusion that the primary gonads of both the oviparous and viviparous oysters show various grades of bisexuality, some individuals being more strongly masculine or more strongly feminine than others in appearance, as indicated by the relative abundance of the cells characteristic of the two sexes. This is likewise true of other pelecypods which normally experience one or more changes of sexual phase, as *Teredo* (Coe, 1935) and *Venus* (Loosanoff, 1936), as well as of certain gastropods, including *Patella* (Orton, 1928) and *Crepidula* (Coe, 1936). In all of these species the initial male phase is occasionally aborted, while other young individuals appear to be so strongly masculine that they have been designated as "true males." In the latter the female phase may be long delayed. Protandry is thus dominant in all the species mentioned but is not exclusive in any of them.

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RENAL FUNCTION IN MARINE TELEOSTS

IV. The Excretion of Inorganic Phosphate in the Sculpin

ALLAN L. GRAFFLIN [1]

(From the Department of Anatomy, Harvard Medical School)

Our knowledge of the nature of the water and salt cycle in the marine teleosts has been markedly clarified by Smith (1930, 1932), who has demonstrated quite conclusively (1) that marine teleosts normally ingest considerable quantities of sea water, which subsequently undergo absorption in the gastro-intestinal tract; (2) that the greater part of the sodium, potassium, and chloride absorbed from the alimentary tract is excreted by some extra-renal route, presumably the gills; and (3) that essentially all of the absorbed magnesium, calcium, sulfate, and phosphate which leaves the body is excreted by the kidneys alone.

The renal excretion of inorganic phosphate in marine teleosts is particularly interesting. It has been shown by Marshall and Grafflin (1933) that the urine of the aglomerular goosefish (*Lophius piscatorius*) may contain large amounts of inorganic phosphate, which is presumably formed in the kidney from some as yet unknown precursor and secreted by the renal tubules. The basis for this interpretation is that injected inorganic phosphate is not excreted either by this fish or by the aglomerular toadfish (*Opsanus tau*). It is to be inferred from this fact that the renal tubules of the glomerular teleost, and perhaps of other animals, would be unable to secrete preformed inorganic phosphate, and that this substance would be excreted only by glomerular filtration. Observations upon frogs (Marshall and Grafflin, 1933) and dogs (Pitts, 1933) support this suggestion. There remains, nevertheless, the possibility that the tubules of the glomerular kidney might, like the tubules of the aglomerular kidney, be able to secrete inorganic phosphate from some occult precursor. Though no evidence in this direction has as yet been adduced for the frog or for mammals, the evidence presented in this paper clearly points to such an operation in the glomerular fish kidney.

Our present experiments were made upon the common sculpin (*My-*

[1] I am indebted to Professor E. K. Marshall, Jr., for his constant advice and criticism during the present investigation, which was carried out at the Mt. Desert Island Biological Laboratory, Salsbury Cove, Maine. I am also indebted to Professor H. W. Smith for critical comments and for assistance with the manuscript.

oxocephalus octodecimspinosus).[2] The study of phosphate excretion in this species has been found to be complicated by two factors: (1) the occurrence of a spontaneous increase in phosphate excretion under experimental conditions; (2) the frequent presence of a precipitate of magnesium phosphate in the urine. Discussion of the experiments upon the mechanism of phosphate excretion, in terms of glomerular and tubular activity, will be deferred until after the consideration of these two complicating factors.

Methods

To determine the rate of urine flow, the bladder was first emptied, with the use of a fine glass catheter, by massage of the overlying abdominal wall. The urinary papilla was then tied off with fine silk thread; and subsequently, at the end of the collection period, the thread was cut and the urine removed by catheter. In this way repeated urine collections could be made upon the same animal. The region of the papilla was always thoroughly cleansed with distilled water and dried before catheterization to avoid contamination. In sacrifice experiments blood was obtained by syringe from the exposed heart (bulbus of aorta); in the course of an experiment it was obtained by syringe from the caudal vessels without exposure (see Grafflin, 1935*a*). Phosphate was determined by the method of Fiske and Subbarow (1925), and is uniformly expressed in terms of millimols per liter. Potassium oxalate was used as anticoagulant. Whenever the urine showed turbidity the precipitate was dissolved with a trace of glacial acetic acid before the phosphate content was determined. The determination of xylose was carried out upon Somogyi (1931) filtrates of plasma and urine. After absorption of the glucose on yeast, xylose was determined by the Folin (1929) modification of the Folin-Wu method. Xylose values are expressed as xylose, not as glucose equivalents, appropriate corrections being made for the diminished reducing power of xylose and its dilution by the water content of packed yeast (see Marshall and Grafflin, 1932).

Observations

Normal Urinary and Plasma Phosphate Concentrations in the Sculpin

Under normal conditions phosphate may appear in the urine of the sculpin in very high concentration, and it is not infrequent to observe a heavy urinary precipitate of magnesium acid phosphate (Pitts, 1934). This phosphate is entirely of metabolic origin, since phosphate is present in sea water in only slight traces. When oliguria is established by obstruction of the gastro-intestinal tract at the pyloric end of the stomach, phosphate continues to be excreted in high concentration (Grafflin and Ennis, 1934). (The oliguria is a consequence of the fact that the animal is dependent upon ingested sea water for its urinary

[2] The nephron in this species consists of a glomerulus, neck segment, brush border segment (the homologue of the proximal convoluted tubule of higher forms) and initial collecting tubule; the brush border segment is bisegmental on cytological grounds (Defrise, 1932; Edwards, 1935).

water.) In the urine obtained from six sculpins immediately after catching on hook and line, Pitts (1934) observed phosphate values ranging from 50 to 155 mM./liter. In six additional freshly caught specimens we have observed values of 7, 40, 113, 130, 140, and 190 mM./liter. Plasma phosphate determinations on freshly caught sculpins gave values of 3.2, 3.4, 3.5, 3.8, 4.4, 4.5, and 5.6 mM./liter. It follows from these observations that phosphate may be highly concentrated, relatively to the plasma, in the urine of normal fish.

Course of Urinary Phosphate Concentration in Sculpins with a Single Daily Catheterization

It was early observed, however, that when individual animals are followed from day to day, with a single catheterization and without tying of the urinary papilla, the urinary phosphate soon drops to low levels and remains there. For example, sculpin No. 14 was followed for twelve days: urinary PO_4 7.0, 20.6, 4.0, 0, 0, 0, 1.5, 0.3, 1.1, 1.7, 1.1, and 1.9 mM./liter. No. 20 was followed for six days: PO_4 149.0, 10.8, 3.6, 2.5, 1.4, and 0.3 mM./liter. The urine flow on the seventh day was 51 cc. per kilogram per 24 hours, PO_4 0.5 mM./liter. This fall in the urinary concentration of phosphate is in part due to diuresis, for it is known that, unless very special precautions are taken, sculpins readily become diuretic under experimental conditions (Grafflin, 1931, 1935*b*; Smith, 1932; Pitts, 1934; Grafflin and Ennis, 1934; Clarke, 1934).

Spontaneous Increase in Urinary Phosphate Concentration and Phosphate Excretion

However, the present investigation has demonstrated a further very significant fact which has been missed in previous studies; namely, that when urine samples are taken at short intervals from a sculpin previously undisturbed (for exceptions see below), the urinary phosphate concentration and the rate of phosphate excretion very often show a moderate or marked, but transitory, increase.[3] Data upon thirteen sculpins are given in Fig. 1. In eleven animals preliminary catheterization (open circles) was followed by a collection period (2 to 3.2 hours), with the urinary papilla tied. Since the phosphate values represent the average concentration for the period, the second points (solid circles) are plotted as of the mid-period. The other two animals (Nos. 70, 72) were

[3] A similar rise was observed in the closely related daddy sculpin (*M. scorpius*), from 0.5 to 44 mM./liter in a short collection period, with the urinary papilla tied. It is to be noted that this species shows marked evidences of glomerular degeneration, some specimens being functionally entirely aglomerular (Grafflin, 1933).

catheterized twice without tying of the papilla, and the second points are plotted at the time of catheterization.

A further group of eleven sculpins which were more extensively studied are recorded in Fig. 2. All of the animals, except No. 37, were studied by catheterization alone, and the actual times of catheterization are plotted (open circles). In the case of No. 37, three collection periods were run successively, and the midpoints of these periods are plotted. All sculpins recorded in Fig. 2 were followed on the second day, and five of them still further on the third day (Fig. 3).

The conditions under which the spontaneous increase in phosphate concentration was observed in these animals are varied.

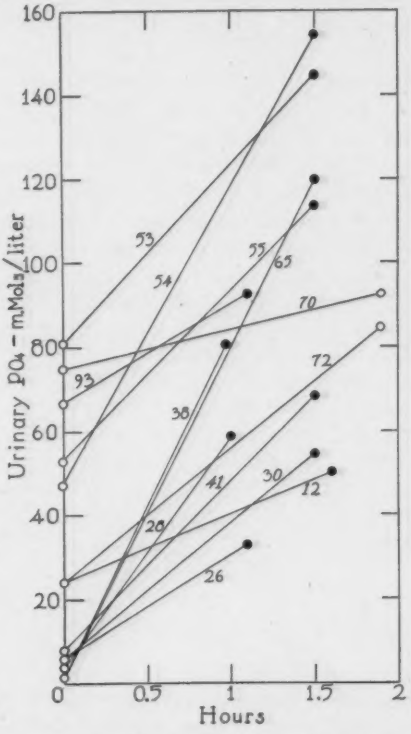

FIG. 1. Spontaneous increase in urinary phosphate concentration in the sculpin. Open circles, catheterization alone. Solid circles, urine collection period with urinary papilla tied.

Fig. 1.—In 11 specimens the rise occurred when the fish were first handled at varying intervals after catching: Nos. 70, 72—day after catching; No. 28—second day; No. 30—fourth day; No. 65—sixth day; Nos. 38, 41, 53, 54, 55, 93—interval indeterminate. (These latter fish were taken at random from the stock supply in a large live car. This stock was added to as fish were procured, and records of individual specimens were not kept.) No. 12 was catheterized once on the eleventh and eighteenth days and the rise was observed on the twenty-third day. No. 26 was catheterized once on the third, fourth, and fifth days, and the rise was observed on the sixth day.

Fig. 2.—In 8 specimens the rise occurred when the fish were first handled at varying intervals after catching: Nos. 47, 48, 49—day after catching; Nos. 43, 44, 45, 46—third day; No. 37—interval indeterminate (stock supply). Nos. 34 and 35 were catheterized once on the third day and the rise was observed on the fifth day. No. 36 was catheterized once on the third and fifth days and the rise was observed on the eighth day.

Figs. 1 to 3 deal only with the increase in urinary phosphate concentration, and a more detailed analysis of the experiments is given in Table I. From the nature of the data given, this table can only include experimental periods in which the urine flow was accurately determined, with the urinary papilla tied. Necessary explanatory considerations for the interpretation of this table are given at the top of p. 365.

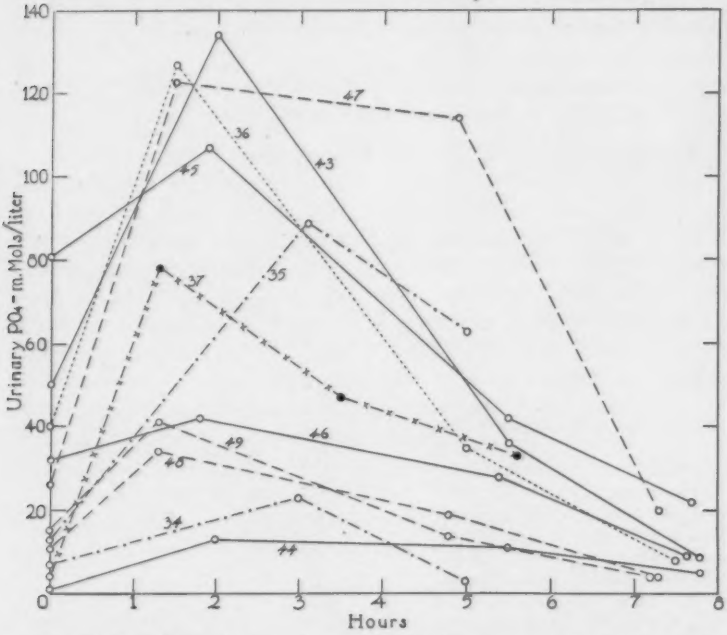

FIG. 2. Spontaneous increase in urinary phosphate concentration in the sculpin. Open circles, catheterization alone. Solid circles, urine collection period with urinary papilla tied. Groups of animals handled identically are graphed similarly (e.g., Nos. 43, 44, 45, 46; Nos. 47, 48, 49).

If, now, we proceed to analyze the data which we have obtained, we are led to the following considerations.

(1) Sculpins very often show a spontaneous increase in urinary phosphate concentration which may be slight, moderate or marked. It must be emphasized that in some specimens it is not observed at all. The degree of consistency with which it occurs is demonstrated by considering groups of sculpins with an identical history and handled identically under experimental conditions (see groups of animals similarly graphed in Fig. 2).

(2) In nineteen of the twenty-four sculpins recorded in Figs. 1 and 2 the spontaneous increase was observed when the animals were first handled, irrespective of the number of days they had been in captivity. The other five animals (Nos. 12, 26, 34, 35, 36) had been catheterized from one to three times previously (see above).

(3) The increase may occur irrespective of the initial concentration (first catheterization) over a wide range (1 to 81 mM./liter—Figs. 1 and 2).

(4) The increase is transitory, in general rising to a peak and then rather rapidly diminishing to low levels.

(5) It seems quite certain from the data available that the increase in urinary phosphate concentration is not associated with an increase in plasma phosphate concentration. Thus, in No. 15 (Table III), in which the urinary concentration showed a spontaneous increase in the first period and just as marked a decrease in the second period, the plasma concentration was considerably higher in the second period (4.2) than in the first (3.1). The rate of phosphate excretion was identical in the two periods. There seems to be no direct relationship between

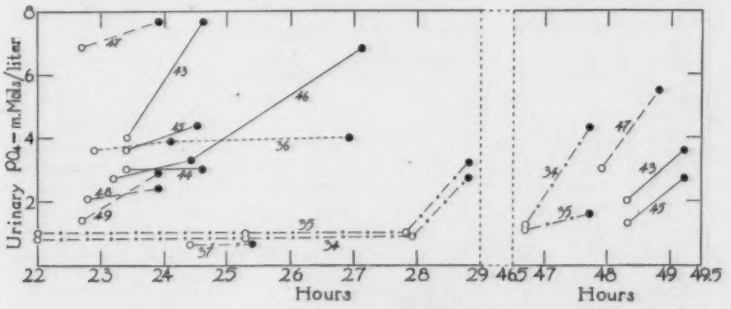

FIG. 3. Subsequent course of urinary phosphate concentration in sculpins recorded in Fig. 2. Open and solid circles, as in Fig. 2.

the absolute level of the plasma concentration and the concentration in the urine (see Table III). Three freshly caught sculpins examined at once gave the following plasma and urinary (in parentheses) phosphate values: 3.2 (190), 3.8 (130) and 4.4 (113) mM./liter.

(6) As discussed above, the computed values for "Increase in PO_4 excretion" (Table I) are almost certainly minimal in most cases. A comparison of the figures for "Increase in PO_4 excretion" with those for "Increase in PO_4 concentration" shows wide discrepancies in the different animals. Obviously, an increase in concentration of 20 mM./liter, with a urine flow of 35 cc., would have exactly the same signif-

TABLE I

Data upon phosphate excretion during urine collection periods in sculpins reported in Figs. 1, 2 and 3.

Sculpin No.	Urine flow	Increase in PO_4 concentration	Additional PO_4 excreted[a]	Increase in PO_4 excretion[b]	PO_4 excretion[c]
	cc. per kg. per 24 hours	*mM./liter*	*mM.*	*mM. per kg. per 24 hours*	*mM. per kg. per 24 hours*
1. *Sculpins reported in Figs. 1 and 2 (No. 37 only)*					
12	7.0	26	0.005	0.18	0.35
26	32.7	27	0.014	0.88	1.08
28	15.4	56	0.015	0.86	0.91
30	16.7	49	0.022	0.82	0.92
37	10.0	74	0.033	0.74	0.78
38	17.2	79	0.043	1.36	1.39
41	26.6	61	0.024	1.62	1.84
53	8.3	64	0.020	0.53	1.20
54	6.8	108	0.027	0.73	1.05
55	9.4	61	0.015	0.57	1.07
65	10.0[d]	119	0.024	1.19	1.20
93	17.0[d]	26	0.008	0.44	1.58
2. *Sculpins on second day of handling (Fig. 3—22 to 29 hours)*					
34	40.7	>1.7	>0.0029	>0.07	0.11
35	49.8	>2.2	>0.0037	>0.11	0.16
36–1	33.2	0.3	0.0002	0.01	0.13
–2	113.6	0.1	0.0002	0.01	0.45
37	68.4	0	0	0	<0.07
43	72.0	3.7	0.0085	0.25	0.55
44	53.6	0	0	0	0.16
45	29.8	0.8	0.0006	0.02	0.13
46–1	61.8	0.6	0.0007	0.04	0.20
–2	73.1	3.5	0.0046	0.26	0.50
47	23.2	0.8	0.0005	0.02	0.18
48	41.5	0.3	0.0005	0.01	0.10
49	59.0	1.3	0.0022	0.08	0.16
3. *Sculpins on third day of handling (Fig. 3—46.5 to 49.5 hours)*					
34	93.4	3.3	0.0066	0.31	0.40
35	142.8	0.3	0.0008	0.04	0.20
43	83.5	1.6	0.0032	0.13	0.30
45	59.8	1.4	0.0015	0.08	0.16
47	11.4	2.5	0.0006	0.03	0.06

[a] Actual volume (in liters) of urine formed in collection period × increase in PO_4 concentration.
[b] Increase in PO_4 concentration × rate of urine flow.
[c] Actual PO_4 concentration of collection period urine (Figs. 1–3) × rate of urine flow.
[d] Approximate.

(1) "Increase in PO_4 excretion" is the difference between the observed excretion rate (last column) and the rate at which PO_4 would have been excreted if the urinary PO_4 concentration had not increased, but remained the same. Actually, the computed values may be considered as minimal values. The calculation is based on the assumption that the urine flow did not increase during the collection period, while in many cases, from the evidence available (Grafflin, 1931, 1935*b*, this paper; Pitts, 1934), it probably did.

(2) "Additional PO_4 excreted" represents the amount of PO_4 which would have to be added to the actual volume of urine formed in the collection period, in order to raise the PO_4 concentration from the level observed at the preliminary catheterization to that observed at the end of the collection period. In order to make clear the significance of this calculation, we must proceed at once to a discussion of the white precipitate frequently noted in sculpin urine. The following quotation is taken from Grafflin and Ennis (1934—p. 287): "It has been the experience of the workers at the Mount Desert Island Biological Laboratory over a period of years that the urine often shows a white precipitate; this is particularly true of the urine in freshly caught fish and of urine accumulated within the first 24 hours after catching. It has further been observed that a clear bladder urine may suddenly precipitate in the catheter or syringe when attempts are made to collect it; or again, the urine may be perfectly clear when collected, but subsequently develop a precipitate when allowed to stand at room temperature. The precipitate can always be dissolved by acidifying the urine with acetic acid."[4]

On analysis Pitts (1934) found the composition of this precipitate to correspond to $MgHPO_4 \cdot 3H_2O$. He makes the following statement: "It is possible that some of the variability of phosphate and magnesium excretion in these fish results from the ejection from the bladder of solid magnesium phosphate, excreted by the kidneys at some earlier time, but remaining in a precipitated state till a later collection period." If this were true, it might offer the entire explanation for the observed increase in urinary phosphate excretion. The significance of the calculation, "Additional PO_4 excreted," now becomes apparent. It represents simply the absolute magnitude of the hypothetical preformed store of phosphatic precipitate in the bladder (or elsewhere) which would be necessary to account for the increase in urinary phosphate concentration noted in the experimental periods.

icance, in terms of "Increase in PO_4 excretion," as an increase in concentration of 100 mM./liter, with a urine flow of 7 cc. Under the circumstances, it is evident that "Increase in PO_4 excretion" is really the fundamental consideration in a given experiment. Calculation from the data given in Section 1, Table I, shows that this quantity varies from 28 per cent to 99 per cent of the total "PO_4 excretion," with an average of 74 per cent.

(7) Since sculpins usually become progressively diuretic when handled repeatedly under experimental conditions, the total phosphate excretion may continue to increase tremendously when the concentration is steadily diminishing. This fact is strikingly demonstrated in the

[4] This precipitate may occur in urines of widely varying PO_4 content. In the present study, on the one hand, cloudy urines (as withdrawn from the bladder) have been observed with a PO_4 concentration as low as 6 mM./liter. On the other hand, perfectly clear urines have been noted which contained as high as 100 mM./liter.

following tabulation (Table II) of the results obtained upon sculpin No. 37 (see Figs. 2 and 3).

TABLE II

Sculpin No. 37; 245 grams	Urinary PO_4	Urine flow	PO_4 excretion
	mM./liter	*cc. per kg. per 24 hours*	*mM. per kg. per 24 hours*
Pretying	4.0	(10.0) *	(0.04)
0–2.5 hours	78.0	10.0	0.78
2.5–4.6 hours	46.5	44.0	2.04
4.6–6.7 hours	32.8	77.4	2.54
24.4 hours (pretying)	1.0	—	—
24.4–26.4 hours	1.0	68.4	0.07

* Assumed maximum.

Causal Factors in the Spontaneous Increase in Phosphate Excretion

It will now be profitable to examine some of the factors inherent in the preceding experiments, in an attempt to determine whether one or more of them might be directly responsible for the observed spontaneous increase in phosphate excretion.

Tying of the Urinary Papilla; Catheterization.—Tying of the urinary papilla can be ruled out at once, since the increase occurs just as readily when sculpins are studied by catheterization alone as when the papilla is tied (Figs. 1 and 2). An evaluation of the factor of catheterization is more difficult. Sculpins are frequently very sensitive to this procedure and struggle vigorously, particularly when the papilla is catheterized for the first time. Usually, on subsequent catheterizations, little or no response is elicited. In certain experiments (Nos. 12, 26, 34, 35, 36—Figs. 1 and 2) the papilla had been dilated by one or more catheterizations previous to the day on which the increase was noted. The intensity of the stimulus in such animals is certainly much less than in animals catheterized for the first time, and one might be led to suppose that the procedure of catheterization is not of itself a significant factor in the observed rise. However, since this procedure is a constant one in the present experiments, it cannot be definitely ruled out.[5]

Pressure; Asphyxia.—That the pressure applied to the abdominal wall in milking the urine out of the bladder plays no rôle is demon-

[5] The evidence is quite conclusive that in the sculpin catheterization is not followed by a shut-down in urine formation (Grafflin, 1935*b*). Even assuming that it did occur, calculation shows the impossibility of explaining the observed increase in PO_4 excretion solely on this basis.

strated by experiments in which the urine was drained into the catheter by gravity alone, without the application of any pressure whatsoever (e.g. Nos. 70 and 72, Fig. 1). For the manipulations of catheterization and tying the fish were usually taken out of water. However, that a short period of asphyxia is of no significance in the observed rise is demonstrated by experiments with the animal under water and breathing normally (e.g. Nos. 30 and 41, Fig. 1).

Phosphatic Precipitate in Urine.—As previously discussed, sculpin urine frequently contains a white precipitate, which Pitts (1934) has shown to be $MgHPO_4 \cdot 3H_2O$. Pitts' suggestion that this precipitate may be retained in the bladder, and so lead to confusion in experiments upon phosphate excretion, has been quoted above. It is essential to determine whether retention of this precipitate, or the presence of phosphatic concretions, in the bladder or elsewhere might be responsible for the spontaneous increase in phosphate excretion which we have observed. With this end in view, the following experiments have been performed.

Bladder Washings.—In 2 sculpins the bladder was emptied by catheter and thoroughly washed out with isotonic salt solution acidified with acetic acid. The papilla was then tied and urine allowed to accumulate for 3 hours. Despite the bladder washing, the urinary PO_4 concentration showed an increase as compared with the pretying urine: No. 62, from 48 to 116 mM./liter; No. 63, from 75 to 100 mM./liter. The total PO_4 recovered from the bladder washings was 0.0015 mM. (No. 62) and 0.0008 mM. (No. 63). Evidence that even markedly turbid urine can be removed by catheter without leaving more than an insignificant trace of PO_4 in the bladder was obtained in 2 experiments on freshly caught sculpins. After preliminary emptying by catheter, the total PO_4 recovered from the bladder by washing as above was 0.0010 and 0.0015 mM.[6]

Ureteral Extractions.—In 3 sculpins the large ureteral trunk visible on the ventral surface of the kidney was dissected out and extracted separately for each animal with acetic acid. Total PO_4 recovery from trichloracetic acid filtrates of the extracts was in each case 0.0010 mM. or less.

Kidney Extractions.—From 4 sculpins the combined posterior and middle kidney was removed and extracted separately for each animal by grinding with washed sand and dilute acetic acid. The anterior portion of the kidney was not used as it is entirely lymphoid in nature. The kidney samples weighed 0.17, 0.25, 0.27, and 0.28 gram, and the total PO_4 recovered from trichloracetic acid filtrates of the extracts was 0.0020, 0.0021, 0.0031, and 0.0026 mM. Urine obtained just before the tissue samples were taken showed PO_4 concentrations of 64, 38, 82, and 93 mM./liter, respectively. Again, the combined posterior and middle kidney was removed from 4 freshly caught sculpins, and the entire sample (1.3 grams) extracted as before. Total PO_4 recovery from the filtrate was 0.0128 mM., giving an average of 0.0032 mM., comparable with the average (0.0025 mM.) of the 4 previous extractions.

[6] In spite of the frequency with which magnesium phosphate is precipitated in sculpin urine, bladder calculi have never been observed. In 456 sculpins (used for another study—Grafflin and Gould, 1936) in which the bladder was opened, calculi were never encountered. To this number can be added several hundred specimens observed in the course of previous studies.

To evaluate these experiments, it is essential to know the order of magnitude of a preformed store of phosphate, either as the usual precipitate or in the form of concretions, which would be necessary to account for such increases in urinary phosphate excretion as we have observed. This calculation has been made for twelve of the animals in Figs. 1 and 2, and appears in Section 1, Table I, as "Additional PO_4 excreted." It varies from 0.005 to 0.043 mM., average 0.021 mM. This quantity is 17.5 times the average phosphate recovery from bladder washings (0.0012 mM.) in the above experiments. In the extreme case (No. 38) it is 36 times this latter quantity. The average hypothetical preformed store is at least 21 times the average recovery from ureteral extractions, and over 7 times that from 8 kidney samples (0.0029 mM.). This factor for the kidney extractions is deceptively low, since we must consider the phosphate contained in the blood, in the renal tissue cells, and in the urine contained in the lumina of the renal tubules and the collecting duct system.

The above experiments justify the conclusion that in the sculpin no significant retention of phosphate, as precipitated magnesium phosphate or as phosphatic concretions, occurs in the bladder, ureter or collecting duct system of the kidney. And we are permitted to conclude, in turn, (1) that the phosphate concentration of urine as withdrawn from the bladder can be accepted as the concentration of the urine as it is elaborated by the kidney; and (2) that the spontaneous increase in phosphate excretion reflects a real increase in renal activity.

In seeking still further for an explanation for the observed spontaneous increase in phosphate excretion, three possibilities were considered.

Muscular Activity.—Sculpins are normally very sluggish. When handled they frequently struggle quite vigorously, to such an extent that to hold them reliably in routine experimental work requires considerable experience. Phosphate compounds are of paramount importance in muscular activity, and muscular exercise in mammals regularly leads to increased urinary phosphate excretion. Under the circumstances, we feel that muscular activity offers the most likely explanation for the sharp increases which we have observed. The significant proportional increases in phosphate excretion observed on the second and third days of handling (Fig. 3, Table I) could be explained on the same basis, but at a much lower level of phosphate available for excretion.

Increased Absorption of Phosphate from the Alimentary Tract.—The phosphate of the body is derived essentially only from the food. Under experimental conditions sculpins develop a progressive diuresis,

associated with a marked increase in the ingestion and gastro-intestinal absorption of sea water. A sudden increase in the absorption of sea water could well lead to rapid absorption of the phosphate contained in the partially digested food present, and so to a marked increase in phosphate excretion. That this mechanism is not fundamental in our experiments is indicated by the fact that some animals were starved for many days before the spontaneous increase was observed. However, it could conceivably play a rôle in animals handled soon after catching.

Reflex Effects upon the Kidney.—While tying of the papilla plays no rôle, reflex effects from the stimulus of catheterization cannot be definitely ruled out as a factor. It is impossible to rule out reflex effects from the handling of the skin. Many injection experiments have shown that liberation of adrenalin in the excitement of experimental handling would not of itself lead to increased phosphate excretion.

The Question of Tubular Secretion of Phosphate in the Sculpin

In approaching the question of tubular secretion, we were guided by the belief of Jolliffe, Shannon and Smith (1932) and Clarke and Smith (1932) that the xylose clearance could be used as a measurement of the rate of glomerular filtration. More recent experiments (Richards, Westfall and Bott, 1934; Shannon, 1934, 1935*a*, 1935*b*; Shannon and Smith, 1935; Van Slyke, Hiller and Miller, 1935*a*, 1935*b*) have shown that there is some reabsorption of xylose by the renal tubules, and that the rate of glomerular filtration can apparently be quite accurately measured in the dogfish, dog and man by the use of inulin. Clarke (1936) has found in the sculpin that the xylose clearance is about 20 per cent less than the simultaneous inulin clearance. But this discrepancy is not of such an order of magnitude as to invalidate the experiments which we had done with xylose in this animal. Our experiments consist of a series of simultaneous phosphate and xylose clearances, as given in Table III. (Clearance $= (U/P)V$, i.e., urine/plasma ratio times urine flow.) Data on the total phosphate excretion in these experiments are included in this table for comparison with the data in Table I. The tubular clearance of phosphate has been calculated by deducting the xylose clearance $\times$ 1.25 (in accordance with Clarke's figures) from the observed phosphate clearance. In a series of observations on normal sculpins with low urine flow, the maximal U/P ratio for xylose observed by Clarke (1934) was 6.5. In our early experiments plasma and urine were removed from three sculpins immediately after catching, and the phosphate U/P ratios were 59, 34 and 26. These data would indicate that the U/P ratio for phosphate in

the sculpin may normally be considerably greater than can be accounted for by the reabsorption of water from the glomerular filtrate. This indication is established as a fact by the data in Table III, where in four experiments direct xylose-phosphate comparisons were carried out. In only one instance did the phosphate clearance approximate the glomerular clearance, after allowing for 20 per cent reabsorption of xylose.[7] A representative protocol for this series of experiments follows.

Sculpin No. 15; 330 grams. July 17. From live car stock at noon; urine PO_4 24 mM./liter. 5:30 P.M., 0.4 gram xylose intramusc. July 18. 8:17 A.M., urine PO_4 13 mM./liter; bladder emptied, papilla tied. 10:13, bled 1.5 cc. 12:09 P.M., urine 0.35 cc.; papilla retied. 2:05, bled 1.5 cc. 4:01, urine 1.25 cc.

TABLE III

Xylose—inorganic phosphate data for the sculpin.

Sculpin No.	Duration of Period	Urine Flow	Plasma		$\frac{U}{P}V$		$\frac{\text{Phosphate}}{\text{Xylose}}$	Tubular Clearance of Phosphate	Total Phosphate Excretion
			Xylose	Phosphate	Xylose	Phosphate			
	hours	*cc. per kg. per 24 hours*	*mg. per cent*	*mM./liter*	*cc. per kg. per 24 hours*			*cc. per kg. per 24 hours*	*mM. per kg. per 24 hours*
10	5.1	42.6	229	2.7	41.3	2096	50.8	2044	5.67
11	5.2	15.8	261	4.0	15.6	337	21.6	318	1.34
15	3.9	6.5	222	3.1	37.7	103	2.7	56	0.32
	3.9	23.3	183	4.2	53.6	75	1.4	8	0.33
17	4.1	8.6	142	2.7	8.6	409	46.4	398	1.10
	4.1	21.5	102	2.7	32.3	957	29.6	917	2.58

The frequency and the magnitude of the observed spontaneous increase in urinary phosphate concentration and excretion make it clear that experiments upon the excretion of exogenous phosphate would be uninterpretable until any significant increase could be adequately ruled out in a given experimental animal. It seems reasonable to suppose that in certain of the animals reported earlier (Nos. 12, 26, 34, 35, 36), catheterized at long intervals, the spontaneous rise may have occurred more than once. The question arose as to whether any significant increase could be suppressed, and the total rate of excretion of endogenous phosphate could be forced to low levels, if a sculpin were followed

[7] It is to be noted that when the sculpin kidney is rendered functionally aglomerular by heavy phlorizinization, phosphate continues to appear in the urine (Marshall and Grafflin, 1932).

closely (i.e., persistently, with repeated catheterizations not separated by long intervals) over a sufficiently long period. Lack of space prohibits a detailed discussion of this aspect of the problem, but the summarized data in Figs. 2 and 3 and Table I indicate that such a condition can be achieved in the sculpin, and that such an animal could be used with confidence in studying the behavior of the kidney toward exogenous phosphate.

In summarizing our observations, we may contrast the aglomerular kidney of the goosefish and toadfish, as studied by Marshall and Grafflin (1933), with the glomerular kidney of the sculpin, as examined here. Both types of kidney excrete inorganic phosphate; the one, where there is no glomerular filtration, the other at U/P ratios so much greater than the simultaneous U/P ratios of xylose that there is no doubt but that tubular activity is involved. The precursor from which the urinary inorganic phosphate is derived in the aglomerular goosefish and toadfish is unidentified, but it is certainly not inorganic phosphate in the plasma. By inference, until evidence to the contrary is adduced, it may be assumed that the tubular secretion of phosphate in the glomerular kidney is an homologous process. Whether the tubules of the glomerular fish kidney can secrete preformed inorganic phosphate cannot be answered from the present data.

The demonstration that the tubules of the glomerular fish kidney can secrete phosphate, presumptively derived from some unidentified precursor in the plasma other than inorganic phosphate, raises the question whether a similar process can occur in the frog or even in the mammal. We know of no evidence at the present time to enable us to answer this question.

Summary

1. When urine samples are taken at short intervals, the urinary phosphate concentration and excretion in the sculpin very often shows a moderate or marked, but transitory, increase. It is demonstrated that this transient increase is not an artifact due to phosphate precipitates in the bladder, ureter or collecting ducts.

2. By repeated catheterization the rate of endogenous phosphate excretion in the sculpin can be reduced to a low level, and any significant spontaneous increase in excretion can be suppressed.

3. It is shown, by a comparison of simultaneous xylose and phosphate clearances, that the glomerular kidney of the sculpin can under certain conditions excrete a urine with a phosphate concentration in excess of that which could be explained by glomerular filtration.

4. In line with Marshall and Grafflin's observations that the aglom-

erular kidney excretes phosphate that is derived from some unidentified precursor (other than inorganic phosphate in the plasma), the present observations establish the presumption that a similar process occurs in the glomerular kidney of the sculpin.

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SOME UNUSUAL CYTOLOGICAL PHENOMENA IN THE SPERMATOGENESIS OF A HAPLOID PARTHENOGENETIC HYMENOPTERAN, ÆNOPLEX SMITHII (PACKARD)

CARL H. KOONZ

DEPARTMENT OF ZOÖLOGY, NORTHWESTERN UNIVERSITY

Since the early part of the century numerous investigators have been concerned with the cytology of haploid animals. For extensive bibliographies pertinent to the cytology of haploid forms the reader is referred to publications by A. Vandel (1931), Franz Schrader and Sally Hughes-Schrader (1931), and Ann R. Sanderson (1933).

More recently Magnhild Torvik-Greb (1935) made a special study of the chromosomes of *Habrobracon juglandis* and also showed that the spermatogenesis of haploid males and diploid biparental males follows the same course. Allan C. Scott (1936) reported haploidy in the males of a coleopteran, *Micromalthus debilis,* and especially emphasized aberrant phenomena associated with spermatogenesis.

The development of the germ cells of the various haploid metazoan groups is much the same. Obviously a convergence or a parallelism is to be expected in view of the fact that no reduction of chromatin is necessary. The salient and conventional features identified with spermatogenesis have been pointed out by many authors. In the first spermatocyte there may be an attempt to recapitulate the reduction division, which is always abortive as regards chromosomes, or the division may be omitted entirely. When division of the cell does take place it is in the form of the pinching off of a non-nucleated cytoplasmic bud. The second spermatocyte division ordinarily is equational, producing two functional spermatids. However, as in the case of *Apis mellifica* (Meves, 1907), the second spermatocyte division may be unequal, producing only one functional spermatid.

Although haploid studies have been concerned chiefly with testing the validity of the concept that males are produced from unfertilized eggs and in confirming the usual development, recently a certain rather unique phenomenon has been reported. Allan E. Scott (1936) found, in haploid parthenogenetically produced males of *Micromalthus debilis,* that a unipolar spindle was regularly produced in the first spermatocyte division. During the first spermatocyte anaphase apparently the chro-

mosomes move away from the unipolar spindle, the movement being independent of the half spindle fibers. The popular idea that spindle fibers are responsible for chromosomal movement is thus shown to be deficient.

It is the purpose of the present paper to describe the spermatogenesis of *Ænoplex smithii*. Emphasis will be placed on unusual cytological aspects which may contribute to our knowledge of the origin of half spindle components and to the morphological and functional reality of half spindle fibers.

Materials and Methods

Ænoplex smithii is the principal ichneumonid hyper-parasite of *Spilocryptus extrematus*, the latter living parasitically on the larvæ of *Samia cecropia*, a moth. The males develop only from unfertilized eggs and are thus produced parthenogenetically.

The original material for this investigation was collected in the vicinity of Chicago, Illinois. The data presented in this paper, however. are based entirely on laboratory specimens reared from virgin females which lay only male-producing eggs.

Male gonads of late pupæ proved to be the most satisfactory for cytological study, showing few gonial and numerous spermatocyte cells. The gonads of females used to secure chromosome counts in the gonial stage were taken from early pupæ. Gonads were dissected out in water and immediately transferred to a fixative, Bouin's fluid being found most favorable. Gonads were dehydrated and cleared in the usual manner, imbedded in paraffin and sectioned at six microns in thickness.

Heidenhain's iron hæmatoxylin (long method) was employed and destaining was done with a saturated aqueous solution of picric acid. When destained sufficiently the sections were exposed briefly to the fumes of concentrated ammonium hydroxide, dehydrated, cleared in xylol and mounted.

The gonads of more than one hundred males treated according to the above method were used as a basis for this study.

Spermatogonia and Chromosome Number of Haploid Males

The paired testes lie dorsal to the intestine in the posterior half of the abdomen. Internally the testes are divided into many cysts, containing cells in the various stages of development.

Testes of very early pupæ show cysts containing only spermatogonial cells. The cells are arranged in the cyst so as to give a compact, rosette appearance. Divisions in gonial cells are few, but are synchronous in each cyst.

It was only from spermatogonial division figures that it was possible to count the chromosomes. The number was established as thirteen (Fig. 1), representing the haploid number of this species.

Chromosome Number of Diploid Females

Polar views of the metaphase of gonial cells taken from late female pupæ show twenty-six chromosomes (Fig. 2). Therefore it is established cytologically that females are diploid and that the males developing from eggs laid by virgin females are haploid.

Spermatocytes and the Maturation Divisions

The testes of late pupæ show few gonial cells but show many first spermatocytes, second spermatocytes, spermatids, and spermatozoa. The first spermatocytes at the end of the growth period form a loose rosette or are scattered indifferently within the cyst. They are considerably larger than late gonial cells. The nucleus of the spherical first spermatocyte (Fig. 3) is prominent and appears reticulated, having one or more poorly defined nucleoli. A prominent cytoplasmic inclusion, which has been referred to frequently as the "Zellkoppel" or "interzonal body," is present in these cells.

In the late prophase the nuclear membrane becomes indistinct and the chromatin material becomes resolved into chromosomes (Fig. 4). At a slightly later stage the spherical spermatocyte has elongated (Fig. 5), the nuclear membrane has disappeared and the chromosomes have massed. A constriction has appeared, partially isolating that portion of the cytoplasm which subsequently will be pinched off as a non-nucleated bud. Indistinct fibers are seen extending from the vicinity of the chromosomal mass into the bud. This stage probably simulates the metaphase of an ordinary mitosis or meiosis. As a rule the bud contains the "interzonal body" (Fig. 9) which is not visible in some of the eosin counterstained preparations (Fig. 6). When the bud is almost completely detached, distinct fibers can be seen extending between it and the nucleated mother cell (Fig. 6). Simultaneously with the pinching off of the bud there arises a monopolar spindle (unipolar or half spindle), the components of which would appear to be formed as outgrowths from the massed chromosomes. The monopolar spindle is cone-shaped with the apex pointing toward the bud; although at first small and inconspicuous, it later becomes progressively larger, elongates and becomes very prominent (Fig. 7).

The massed chromosomes and their attached half spindle components next separate into two approximately equal halves (Figs. 8–9).

Simultaneously with this separation and with the spindle elongation there appears to be distinct anaphasic movement on the part of the chromosomes. At the completion of this anaphasic movement the chromosomes, with the attached spindle fibers trailing, have traversed the cell and occupy that part of the cell distal to the locus of the formerly attached bud (Fig. 10).

There is no formation of a nuclear membrane between the first and second maturation divisions. The telophase chromosomes gather into a dense compact mass during an abbreviated interkinesis. In the formation of this interkinetic mass the chromosomes, preceding, apparently drag their attached fibers with them (Fig. 11), so that in early interkinetic masses the fibers of the monopolar spindle radiate from the mass (Fig. 12). In later interkinesis the spindle fibers completely disappear (Fig. 13).

New spindle fibers identified with the second spermatocyte division soon make their appearance. The fibers, a number of which constitute the bipolar spindle, from all appearances are formed as outgrowths from some material contained within the interkinetic chromosomal mass. The components of each half spindle comprising the bipolar spindle are at first darkly staining short extensions from the chromosomal mass (Fig. 14) but subsequently are less basophilic and become progressively longer (Fig. 15). The components of each half spindle very early assume a cone formation. The cones, however, do not grow out in opposite direc-

EXPLANATION OF PLATES

All figures are camera lucida drawings drawn to the same scale. They were originally drawn to represent a magnification of 4,807 and have been reduced one-third as represented here.

EXPLANATION OF PLATE I

FIG. 1. Side view of spermatogonial metaphase showing thirteen chromosomes, the haploid number.

FIG. 2. Polar view of female gonial cell showing twenty-six chromosomes, the diploid number.

FIGS. 3–4. First spermatocytes, early and late prophase respectively.

FIGS. 5–6. First spermatocytes showing the pinching off of the cytoplasmic bud and the formation of the monopolar spindle.

FIG. 7. Second spermatocyte showing prominent monopolar spindle.

FIGS. 8–10. Second spermatocyte showing anaphasic movement of the chromosomes, with spindle fibers trailing.

FIG. 11. Chromosomes of second spermatocyte, with spindle fibers trailing, gathering to form interkinetic mass.

FIG. 12. Second spermatocyte with half spindle fibers radiating from interkinetic mass.

FIG. 13. Interkinetic mass. Half spindle fibers have disappeared.

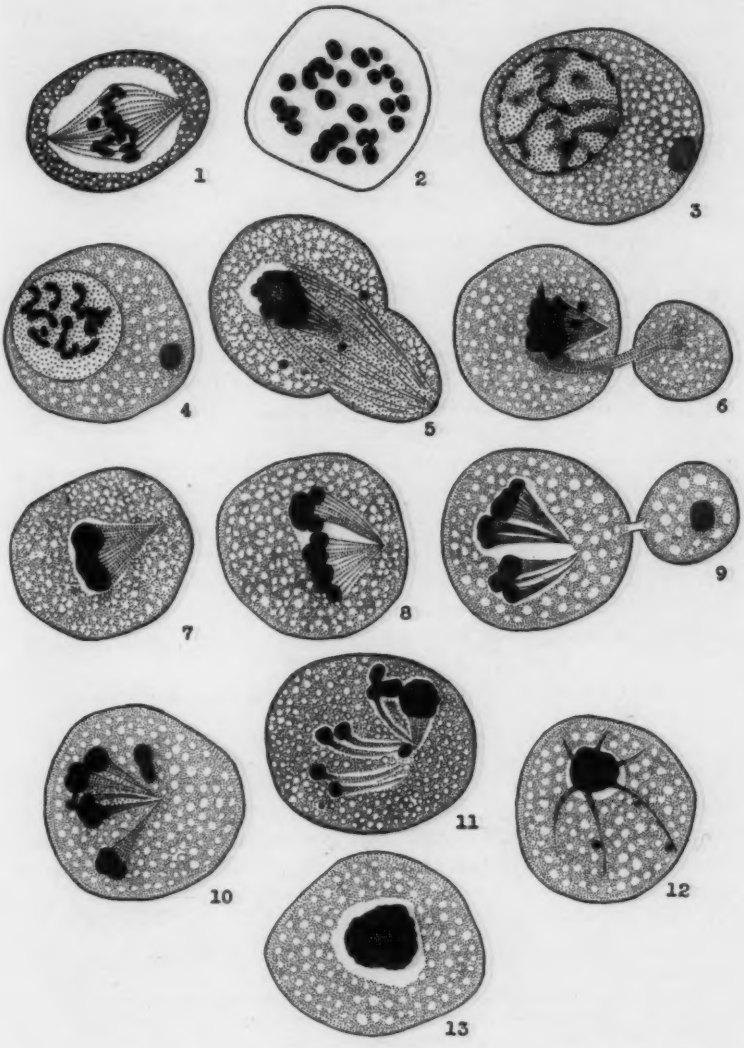

Plate I

tions in the same plane. On the contrary, both cones not infrequently appear to grow out in the same general direction and in forming the bipolar spindle in which the two halves are opposed in the same plane (Fig. 17), each half cone may be generally figured as describing an arc of about ninety degrees. In the course of such movement many crescentic figures may be seen (Figs. 14–16).

The Spermatids

The second spermatocyte division may be equal, producing two normal spermatids (Figs. 18–19). Frequently, however, when the division has proceeded to late anaphase, it is suppressed (Fig. 22) and the cytoplasm of the two potential spermatids, instead of separating, fuses and an aberrant spermatid is formed. This spermatid is twice the size of the normal spermatid and is at first binucleate (Figs. 23–25). Later the two nuclei of the aberrant spermatid fuse forming a single large nucleus (Fig. 26).

In early spermiogenesis the metamorphosis of the normal spermatids (Figs. 20–21) and the aberrant spermatids (Figs. 27–28), both types of which may be found within the same cyst, appears to be similar except for the distinct dimorphism in size. Dimorphism in size of sperm has not been observed and therefore no evidence is available that would suggest that these large spermatids metamorphose into functional spermatozoa. It would seem likely that they are pathological in character.

Unusual Cytological Phenomena and Possible Significance

Monopolar Spindle

Spindles which normally have only one pole have been reported recently by several authors. Metz (1933) described a monocentric mitosis which normally effects a regular and precise segregation of chromosomes in the first spermatocyte in *Sciara*. Sally Hughes-Schrader

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Explanation of Plate II

Fig. 14. Origin of bipolar spindle from interkinetic chromosomal mass of second spermatocyte.

Figs. 15–16. Crescent figures of second spermatocyte prophase.

Fig. 17. Metaphase of second spermatocyte division.

Figs. 18–19. Normal spermatids.

Figs. 20–21. Early spermiogenesis of normal spermatids.

Figs. 22–25. Binucleated aberrant spermatids.

Fig. 26. Large mononucleated spermatid, the nucleus being formed by the fusion of two nuclei.

Figs. 27–28. Early spermiogenesis of aberrant spermatid.

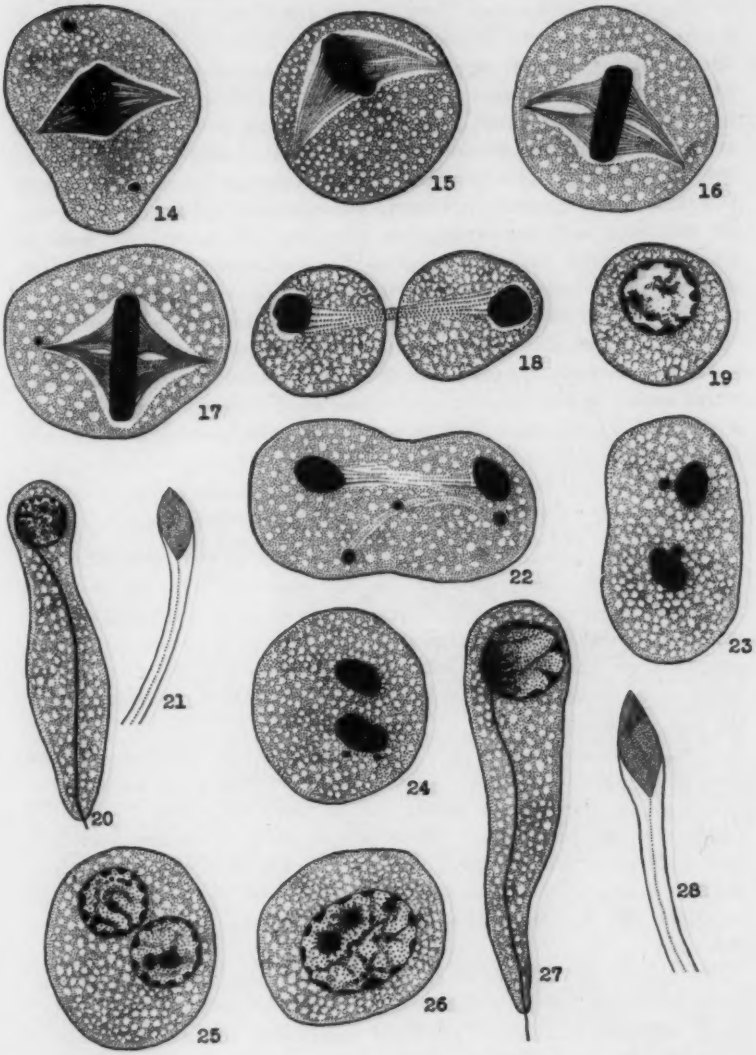

Plate II

(1935) reports half spindle formation in connection with the condensed group of chromosomes in the germ cells of *Phenacoccus acericola*, reduction being effected by the consequent passage of the condensed group to the single pole of the spindle. Scott (1936) found that a unipolar spindle is regularly formed in the first spermatocyte division of a haploid parthenogenetically produced beetle, *Micromalthus debilis*. The monopolar spindle described in connection with the present work is similar in its general features to those already reported. They are all identified with the maturation divisions. However, the spindles reported by Metz and Sally Hughes-Schrader are found in diploid animals. The monopolar spindle here described and the one reported by Scott are found in the first spermatocyte of haploid animals, and therefore are in particular agreement as to time and place of formation.

It is commonly assumed that spindle fibers are in some way associated with a bipolar tension. In the light of these monopolar spindles it would seem that spindle fibers may exist entirely independent of any bipolar condition.

Functional Reality of Half Spindles

Anaphasic movement of chromosomes such as that described here presents an interesting problem. It has generally been assumed that whatever the nature of the half spindle may be, it is concerned with, if not responsible for, this anaphasic movement. Quite obviously, the anaphasic movement here described in connection with the first spermatocyte is in no capacity identified with, and is entirely independent of, the half spindle fibers.

The movement of these chromosomes is no doubt something like that described by Metz (1933) for *Sciara* during the first spermatocyte segregation of chromosomes or similar to the movement reported by Scott (1936) for the chromosomes during the anaphase of the first spermatocyte division in *Micromalthus debilis*. Metz indicates that the movement in *Sciara* is an " autonomous " movement on the part of the chromosomes, and that the half spindle fibers could exert only a retarding influence.

In view of these cases where half spindles play no part in the anaphase movement of chromosomes, it is quite obvious that any theory which attempts to attribute the force responsible for the chromosomal movement to half spindles or their components is not correct in its entirety.

Morphological Reality and Origin of Half Spindle Fibers

Some evidence exists for the morphological reality of half spindle fibers. Perhaps the most convincing evidence, as pointed out by Schrader (1934), pivots on the simple argument that if the half spindle or their components can be made to bend, it is evidence for their morphological reality. Schrader noted that metaphase half spindles or their components undergo bending and distortion in centrifuged cells, and in view of this experimentally produced evidence concluded that the half spindle components have morphological reality.

If bending and distortion of half spindles or their component fibers is evidence for their morphological identity, then perhaps the evidence afforded by the present study is most convincing. In the first place extreme bending of fibers is observed when the telophase chromosomes gather to form the interkinetic mass (Figs. 11–12). The chromosomes preceding drag their attached fibers with them so that in early interkinesis they radiate from the chromosomal interphase mass. In the second place the bipolar half spindles of the second spermatocyte division, in becoming oriented so that the two halves are opposed in the same plane, exhibit considerable bending (Figs. 14–16) as evidenced in the crescentic figures. The phenomena are even more convincing when it is realized that they are natural phenomena and not artificially produced.

Perhaps further evidence for the morphological reality of half spindle fibers is forthcoming in view of the apparent manner in which the spindle components arise. Carothers (1934) has especially emphasized that half spindle fibers are of chromosomal origin. In the present study it appears that the monopolar spindle of the first spermatocyte and the bipolar spindle of the second spermatocyte arise as outgrowths of some substance contained within the chromosomes. If the spindle fibers are outgrowths of the chromosomes, this would appear to be evidence *per se* that they do have morphological reality.

Summary

The number of chromosomes as demonstrated by the cytological study of gonial cells is thirteen in the male and twenty-six in the female. Experimentally it was found that virgin females gave rise only to males. In view of the experimental and cytological evidence it is concluded that males having the haploid chromosome number develop from unfertilized eggs and are thus produced parthenogenetically.

The pinching off of a non-nucleated cytoplasmic bud characterizes the first meiotic division.

A monopolar spindle is regularly formed simultaneously with the pinching off of the first spermatocyte bud. This spindle would appear to be of chromatin origin.

The chromosomes or chromosomal masses, with spindle fibers trailing, exhibit pronounced anaphasic movement identified with the first spermatocyte division. The force responsible for this anaphasic movement apparently is entirely independent of the half spindle fibers.

There is no formation of a nuclear membrane between the first and second spermatocyte divisions. However, after their characteristic anaphasic movement the chromosomes gather into a compact mass for an abbreviated interkinesis.

The tendency for the half spindle components to be dragged about by the chromosomes during the anaphase of the first spermatocyte division and during the movement of the chromosomes in the formation of the interkinetic mass would seem to indicate that half spindle fibers have morphological reality.

The bipolar spindle identified with the second spermatocyte division appears to originate as an outgrowth from the interkinetic chromosomal mass. This would seem to be evidence for morphological reality of the fibers.

The second spermatocyte division may be equal, producing two normal spermatids.

Not infrequently aberrant spermatids are formed as a result of the secondary fusing of the parts of a partially divided second spermatocyte. They have a single large nucleus which is formed as a result of the fusion of two normal nuclei.

In early spermiogenesis the development of the two types of spermatids appear to be similar except for differences in size.

At present no evidence is available to indicate that the aberrant spermatids develop into functional spermatozoa.

ACKNOWLEDGMENT

The writer wishes to express his indebtedness to Dr. C. L. Turner for advice and assistance during the course of this work.

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PROGRAM AND ABSTRACTS OF SCIENTIFIC PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY

JUNE 30, 1936

The effect of methylene blue on the spectrophotometric picture of hemoglobin, CO-hemoglobin and CN-hemoglobin. Matilda M. Brooks.

The purpose of these experiments was to find out the effect of injections of methylene blue on the spectrophotometric picture of hemoglobin (hb) and of CO-hb and CN-hb in rabbits. The blood was taken by heart puncture, diluted to 1 per cent with .4 per cent NH_4OH and then analyzed immediately in the spectrophotometer. The extinction coefficient at wave lengths 540/560 $m\mu$ = R were determined. These values indicate the percentage of oxy-hb present.

There were three main results found; the absorption maximum of the CN-hb curve and the curve for the blood containing methylene blue were identical with that for oxy-hb; the time curve for blood containing CO and methylene blue showed 100 per cent oxy-hb within a short time. There was no difference in the absorption of oxy-hb and CN-hb in the region of the u.v. spectrum but the absorption was greater in the case of oxy-hb than of CN-Hb when wave lengths from 7300 to 10,300 Å were used. Eggert, using the infra-red region, found that blood containing methylene blue gave the same spectrum as oxy-hb while that for blood subjected to CO even after 45 minutes aeration, gave the spectrum of CO-hb.

The conclusions are as follows: the evidence for the change of CO-hb to oxy-hb by the action of methylene blue, *without invoking the methemoglobin formation theory* seems unequivocal. This may be caused by a catalytic action on the part of methylene blue, or it may be due to a poising effect on the oxidation-reduction potential of the system making it compatible with the re-formation of oxy-hb.

The phosphatase content of the developing chick embryo. Harry J. Lipman.

The phosphatase activity of the white, of the yolk and of the embryo were determined in eggs of white Leghorn hens. The determinations were made in intervals of three days during the process of incubation. For the determinations the Brigg's modification of the Bell Doisy colorimetric method for the determination of inorganic phosphorus was used. In the technique the amount of inorganic phosphate formed by the enzyme activity is measured and calculated in total milligrams (absolute values) and in milligrams per gram of wet body weight (relative values).

The absolute values and the relative values for the whole egg at zero days incubation are 0.487 mg. phosphorus and 0.082 mg. phosphorus respectively. Both of these values rise to two maxima, one at six days and the other at fifteen days of incubation, and in doing so run in a parallel fashion. The relative phosphatase values for white, yolk and embryo and the absolute values for white and yolk show these same two maxima. However, the absolute data for the embryo give a fairly good S-type curve. The steepest part of the curve coincides with the period of high ossification activity in the embryo. When the absolute and relative values for the embryo are plotted together on the same graph, the drop of the relative values after the fifteenth day stands out conspicuously against the continuous S-type rise of the absolute values. This drop can be interpreted by the fact that from this day on the

growth of the embryo proceeds at a very rapid rate with which the increase in phosphatase activity does not hold pace. It was found that the material used had a high coefficient of variation. This was also true for the experimental data. These facts make the results less significant than was expected.

The description of an oxidative process maintaining the frequency of the heart beat. Kenneth C. Fisher and Laurence Irving.

Oxidation-reduction potentials and potentiometric determination of ascorbic acid. Eric G. Ball.

JULY 7

Nerve cells without central processes in the fourth spinal ganglion of the frog. Alfred M. Lucas.

Serial sections of the fourth spinal nerve of the bullfrog which included the roots, spinal ganglion, dorsal rami, spinal nerve trunk, ventral ramus, communicating ramus, sympathetic trunk anterior to the fourth nerve and the celiac nerve which were stained in toto with osmic and with silver methods, furnished fiber counts in the regions mentioned. The number of myelinated fibers found in the two roots was 667 and about 259 of these passed to the dorsal rami leaving 408 fibers for the spinal nerve, but distal to the ganglion 950 myelinated fibers were counted. Total fiber counts from silver preparations revealed 1,372 in the dorsal root, 648 in the ventral root and 883 subtracted passing to the dorsal ramus left 1,137 for the spinal nerve. Actual count of the spinal nerve showed 5,277 fibers. The additional fibers arose from the dorsal root ganglion in which were located 5,220 cells. It is concluded, therefore, that some 3,500 cells are present in the spinal ganglion which lack central processes passing through the dorsal root to the cord. The distal processes pass through the communicating ramus to the celiac nerve. Other interpretations have been considered, namely, that dorsal root fibers might have branched in the ganglion, that during development the sympathetic ganglion failed to migrate peripherally as expected, and that the missing central processes had been overlooked when counts were made on the dorsal root.

Receptor areas in the venæ cavæ and the pulmonary veins and their relation to Bainbridge's reflex. José F. Nonidez.

Extinction of reflex responses in the rat. C. Ladd Prosser.

Factors influencing the electrical activity of the brain. R. W. Gerard.

JULY 14

The permeability of the erythrocytes of the ground-hog. A. K. Parpart.

The red cells of the ground-hog have been found to be very much more permeable to a variety of dissolved substances than those of any other species thus far studied. The penetration of non-hemolytic non-electrolytes from isosmotic solutions was followed by the hemolysis method, using photoelectric recording. A few typical results are given below, the figures indicating the time in seconds required for 75 per cent hemolysis in 0.3 M solutions at 20° C.: ethylene glycol, 1.9; glycerol, 2.2; diethylene glycol, 3.0; triethylene glycol, 5.3; erythritol, 5.8; sorbitol, 59.0; mannitol, 80.0; d xylose, 133; d-l arabinose, 256; glucose, 1080; propyl alcohol, 1.4; α, β dioxypropane, 1.9; α, γ dioxypropane, 4.5; water, 0.9. The permeability of these cells to glycerol is only slightly reduced by $NaHCO_3$, Cu^{++}, and CO_2. The red cells of the

ground-hog combine the permeability characteristics of those of man and the mouse. Those of man are known to be permeable to sugars but not to polyhydric alcohols such as mannitol, the reverse is true in the mouse, while the ground-hog erythrocytes are readily permeable to both of these types of substances.

Effects of salts on the injury potentials of frog's muscle. H. Burr Steinbach.

Variations in injury potential were noted as the salt content of the fluid bathing the uninjured portion of the frog sartorius was varied between the limits 0.1 molar to 0.0005 molar. When the observed potential differences are plotted against logarithms of concentration of salt applied, regular curves result. The shapes of these curves can be predicted by the equations for diffusion potentials for such salts as NaCl and $NaC_2H_3O_2$. With high concentrations bathing the uninjured surface the slope of the curve is characteristic of the salt applied. With low concentrations the slope of the curve is determined by the electrolytes of the tissue and is nearly independent of the nature of the salt in the solution. With KCl solutions, the p.d/ log concentration curve is as calculated for concentrations less than 0.01 molar. With concentrations 0.01 molar or greater, the observed potentials become increasingly negative to the calculated as the KCl concentration increases. With isotonic (ca 0.1 molar) KCl, the difference between observed and calculated values is 40 to 50 millivolts, or nearly equal to the injury potential itself. A value of 40 to 50 millivolts must be added to calculated values in order to obtain a quantitative agreement with the observed values for NaCl, $NaC_2H_3O_2$ and the dilute range of KCl. It is concluded that the presence or concentration of such indifferent salts as NaCl and $NaC_2H_3O_2$ (when they are applied to the uninjured surface) has little effect on the injury potential. Potassium, on the other hand, in concentrations 0.01 molar or greater, acts on the uninjured surface to lower the measured injury potential, probably by producing "injury" at the point of application. The concentration at which KCl first shows specific effects is about that at which KCl contracture sets in.

Free calcium in the action of stimulating agents on Elodea cells. Daniel Mazia and Jean M. Clark. (The full paper appears in this issue of the BIOLOGICAL BULLETIN.)

A photoelectric method for recording past chemical reactions. Application to the study of catalyst-substrate compounds. Kurt G. Stern and Delafield DuBois.

JULY 21

Observations on lens regeneration in Amblystoma. W. W. Ballard.

In the literature, *Amblystoma mexicanum* is said to be capable of Wolffian lens regeneration at all stages in its life history. It is found that *Amblystoma punctatum* loses this ability very rapidly in late embryonic stages. At stages 38–39, lenses are formed and separated from the iris in 4 days in 100 per cent of cases. At stage 43, six days later, practically no lens regeneration is obtained. Similar results were gotten from embryos of *A. tigrinum*, and *A. microstomum*. During larval stages of *A. punctatum*, *A. tigrinum*, *A. microstomum*, *A. jeffersonianum* and *A. opacum*, no lens regeneration was obtained over periods up to four months after extirpation.

Using uniform material, a greater percentage of *A. punctatum* embryos regenerated lenses in light than in darkness, at 5° C. than at room temperature, in 6/8 Ringer's solution than in 1/8 Ringer's.

Parabiotic twins were formed in *Amblystoma punctatum* at stages 38–41 in which one lens lay in the pupil between two eyeballs whose irises were superimposed.

Of these two eyeballs, the one which had previously been deprived of its lens regenerated a new one, in spite of the presence partly within it of the lens of the other eye.

Fertile eggs from pheasants in January by night-lighting. T. H. Bissonnette.

A quantitative analysis of the anterior pituitary-ovulation relation in the frog. Roberts Rugh.

Since the original work by Dr. O. Wolf in 1929, ovulation has been induced in an increasing variety of amphibia. In 1934 it was first suggested that while either male or female glands could be used to induce ovulation, there was a potency difference favoring the female anterior pituitary. Since the response to anterior pituitary injection was not uniformly dependable, it seemed desirable to study this relationship quantitatively.

The work was divided into two phases: first, an attempt was made to determine any correlations between sex, body weight, body length, gonad weight, and anterior pituitary weight. The second phase was a study of the degree of ovulation elicited by quantitatively determined doses of the gland.

Over 700 frogs were used, covering two periods: November and February. These months were chosen as representing the beginning and the end of the normal hibernation period. Since it was found that anterior pituitaries varied in weight from 0.6–1.6 mgm., in order to lessen observational error the glands were removed from frogs of approximately the same body length and were weighed in groups of twenty.

While the body lengths of males and females averaged almost the same for November and February, there was consistent reduction in average body weight and average gonad weight, over this hibernation period. Body length, as measured from nares to cloacal opening, was the standard by which other variables were considered. It was found that males and females with body lengths less than 71 mm. were immature; that those between 71–74 mm. showed the beginning of sex differentiation and gonad growth; and males and females longer than 74 mm. showed sex differences in the relative weights of their anterior pituitaries. These differences favored the males, both in November and in February. Over this hibernation period there was considerable reduction in relative weight of the anterior pituitaries in both sexes, at some stages this was as much as 29 per cent. There is a similar reduction, over the hibernation period, of relative ovarian weights. If anterior pituitary weights are plotted against ovarian weights, it is demonstrated that there are correlating reductions in the weights of these two organs, all points falling on a straight line for both November and for February.

In respect to the induction of ovulation, it was found that in November 8 mgm. of male anterior pituitary induced about 42 per cent of the eggs to leave the ovaries while 5 mgm. of female gland tissue induced 85 per cent ovulation. In February 4 mgm. of either male or female gland tissue would induce 100 per cent ovulation, and in doses less than this there were indicated differences in potency favoring the female gland. These seasonal differences may be due to hibernation concentration of the donor's anterior pituitary; to greater susceptibility of ovaries in February than in November to the anterior pituitary hormone; or even to the activation in this prebreeding period of the recipient's own anterior pituitary.

The anterior pituitaries removed from females which had been induced to ovulate showed no decrease in potency in respect to inducing ovulation in other females. This supports the thesis that the injection of the hormone is comparable to the liberation of the host's hormone, and that the host's hormone is in no way affected.

If frogs are selected at random, it will be found that the average male anterior pituitary is about 16 per cent heavier than that of the average female, but is only

60 per cent as potent in respect to inducing ovulation. This supports the thesis that the female glands are approximately twice as potent as the male glands.

Like many biological processes, which at first seem to be simple, this relationship between the anterior pituitary and ovulation resolves itself into a number of variables: (1) size and sexual maturity of the donor of the anterior pituitary; (2) concentration of the hormone through hibernation reduction in weight; (3) activity of the recipient's own gland; (4) source of the hormone, i.e., from male or from female; (5) dose of the hormone (mgm. of gland tissue); (6) size and sexual maturity of the recipient; and (7) size and susceptibility (maturity) of the ovaries to ovulation induction.

This quantitative study points toward seasonal as well as metamorphic changes in the anterior pituitary which must be studied from the cytological point of view. This study is at present being made on the bullfrog, *Rana catesbiana*, and will be reported subsequently. (This paper will appear in full in the January, 1937 issue of *Physiological Zoology*.)

Experimentally induced coupling in the toad, Xenopus lævis. (Moving picture.) H. A. Shapiro.

JULY 28

The relation between vitamins and the growth and survival of goldfishes in homotypically conditioned water. Gertrude Evans.

A possible endocrine rôle of the eosinophil leucocytes in the female rat. C. P. Kraatz. (The full paper will appear shortly in the *American Journal of Physiology*.)

An interpretation of the secondary lymphoid nodules in the albino rat. J. E. Kindred.

Quantitative data from a detailed study of serial sections of submaxillary, inguinal and mesenteric lymph nodes, and Peyer's patches of eight 80-day old albino rats from the same litter, are presented in support of Röhlich's view that the secondary lymphoid nodule is both a germinal center and a center of reaction to substances in the blood stream.

The secondary nodules are prolate spheroidal or spherical in shape and each is roughly divided into a light and a dark zone which differ quantitatively from each other in respect to rates of mitosis, incidences of macrophages, and the several varieties of lymphocytes. The light zones are capped externally by a cup-shaped layer of small lymphocytes and reticulum. The blood capillary plexus of the nodule lies in the light zone.

Because the dark zone has a statistically significant higher rate of mitosis than the light zone in all nodules studied (e.g. mitosis per 1,000 cells with standard error of mean, submaxillary nodes: dark zone 31 (2.3); light zone, 6.3 (0.3)), it is concluded that the dark zone is the germinal center of the nodule. On the other hand, the presence of a blood capillary plexus, approximately equal incidences of small lymphocytes (e.g. 54 per cent (1.6) in mesenteric nodes; 60 per cent (0.6) in Peyer's patches) and macrophages containing degenerating nuclei of small lymphocytes (e.g. 52 (6.3) per 1,000 cells in mesenteric nodes; and 42 (2.3) in Peyer's patches) in the light zones of the nodules of both lymph nodes and Peyer's patches, suggests that the functional activity of the light zone is related to the local reaction of small lymphocytes to substances in the blood. The quantitative data suggest that the minimum mitosis rate necessary to maintain this relation is the rate in the dark zones of Peyer's patches (e.g. 15.5 (0.5) per 1,000 cells). The close relation between the nodules of the lymph nodes and the lymphatic channels, together with the higher rates of mitosis in the

dark zones (e.g. submaxillary nodes, 31 (2.3); inguinals, 32 (2.4); mesenterics, 30 (2.5) per 1,000 cells) suggest that not only do the dark zones of the nodules of the lymph nodes function to maintain a local balance of small lymphocytes, but in addition contribute small lymphocytes to the lymph stream.

From the fact that medium-sized lymphocytes predominate in the dark zones (e.g. submaxillary nodes; dark zone 80 per cent; light zone, 40 per cent) and are the cells most frequently found in mitosis (e.g. submaxillary nodes; 90 per cent of cells in mitosis are medium-sized lymphocytes), it is concluded that the medium-sized lymphocytes are the lymphoblasts.

Physiological adjustments to diving in the beaver. Laurence Irving.

When a normal beaver ceases breathing during submersion, its heart is inhibited. During periodic breathing, anesthetized beaver showed slowing of the heart and a greater than fifty per cent fall in arterial blood pressure during the intervals between breathing movements. A similar fall in blood pressure, slowing of the heart and inhibition of breathing movements followed from gentle inflation of the lungs of the beaver. In addition it was observed that the blood flow through the hind leg was nearly arrested when the lungs were inflated. It was not obvious to what extent constriction of the vessels in the leg contributed to the retarded blood flow, but other observations indicated that the peripheral blood vessels were able to change blood flow rhythmically. Apparently the depressor effect of inflation of the lungs is the same in the beaver as it is in other animals. The difference in the beaver is in the accentuation of the response, which easily arrests breathing for a minute, slows the frequency and decreases the force of the heart and very nearly arrests blood flow through the muscles. These responses are part of the respiratory adjustment of diving animals for enduring asphyxia. It is suggested that the stimulus of inflation of the lungs occurs at the start of a dive and brings about this sequence of physiological adjustments which prepare for asphyxial conditions before the chemical stimuli are effective. The responses seen in the beaver following inflation of the lungs are like those of other mammals except that they are quantitatively greater.

August 4

Development of Arbacia eggs without nuclei: parthenogenetic merogony. Ethel Browne Harvey. (The full article appeared in the August, 1936 issue of the Biological Bulletin, vol. 71, pp. 101–121.)

Cortical changes in Arbacia eggs during fertilization—a moving picture. F. Moser.

Temperature effects on mitotic changes in Arbacia eggs. H. J. Fry.

Sea-urchin larvae with cytoplasm of one species and nucleus of another. Sven Hörstadius. (The full report has already appeared in *Mem. Mus. d'Hist. nat., Bruxelles*, Ser. 2, vol. 3.)

August 11

A study of the cells of the adrenal gland of the ewe during estrus and pregnancy. Laura J. Nahm.

Studies in calcification: III. The shell of the hen's egg. E. Alfred Wolf and Grace Riethmiller.

In two previous papers by McBride and Wolf it was shown that the Gomori modification of the silver nitrate method can be successfully applied to the study

of early stages of calcification of bone and teeth. Since these structures are composed mainly of calcium phosphate, it was desirable to test the method on calcified structures that are predominantly carbonates; the shell of hen's egg was selected in which 89–97 per cent is calcium carbonate and only 0.5–5 per cent calcium phosphate.

The eggshell shows three layers: a thin, hard, structureless outer membrane, the main calcareous body and an inner fibrous layer into which dip the calcified nipples of the main body. After treatment the shells were dark on both surfaces showing where the silver nitrate had reacted, but the main body of the shell was still white and hard; decalcification therefore was required. Cross sections through the main part of the shell showed no traces of silver, except for a brown color of the surface membrane. The fibrous membrane under low power magnification had the appearance of a leopard skin: a large number of black spots were more or less evenly scattered over the area of the membrane, the fibers of which were light brown in color. Under high magnification (oil) it could be seen that the spots were due to a precipitation of silver within the fibers themselves. This is taken as an indication of the location of the calcium phosphate deposits in the eggshell.

The failure of the calcium carbonate to react in the case of the eggshell would then suggest that our description of the process of calcium deposition in bone and teeth of rat embryos, as it was given last year, was incomplete since it did not include the carbonate portion (6 per cent) of the calcifying structures.

Diffraction patterns of striated muscle and sarcomere behavior during contraction. Alexander Sandow.

A method has been devised that permits photographically recording the diffraction patterns formed by 1 mm. long segments (i.e., about 400 striations) at different positions along the length of the frog sartorius (*Rana pipiens*) over the course of single contractions. The simultaneously produced myograms are included on the records.

The patterns produced by a set of different segments of the sartorius undergoing isometric twitch, supermaximally stimulated at *in situ* length, include the following: all records have 1st order spectra of practically unchanged intensity over the whole course of the twitch; the displacements of the 1st order spectra produced by the segments of the tibial two-thirds of the muscle indicate that, in relation to resting length, the corresponding sarcomeres are shortening, while the patterns of the pelvic-third segments show that here the sarcomeres are being stretched. The nature of the shortening and stretching of the sarcomeres at the various muscle levels indicates that the contraction wave is initiated at the nerve plexus and then spreads up and down the muscle away from this region.

During supermaximally stimulated moderately loaded isotonic contractions the patterns show 1st order spectra similarly unchanged in intensity, but with very great displacements, indicating sarcomere shortening up to 20 per cent; contraction, however, takes place at all levels of the muscle.

During both isometric and isotonic contractions the 0th order increases, and the 2nd order decreases in intensity.

The above results, particularly the intensity variations, are interpreted to mean that Jordan's description of contracting sarcomere behavior (*Physiol. Rev.*, **13**: 301, 1933) does not hold for the frog sartorius; but that in the contracting frog sartorius: (1) a sarcomere, in the sense of consisting of a Q band flanked at each end by one-half of each of the adjoining I bands, remains intact, although (2) it may contract or be stretched depending on its position in the muscle and the nature of the contraction; and (3) there occurs an unequal change in the refractive indices of the Q and I substances, or a variation in the ratio of the lengths of Q and I bands, or both of these modifications. And whatever the directions of these changes for contraction of sarcomeres, they are probably opposite in direction for stretch.

The effect of some oxidation-reduction indicator dyes (phenol indophenol) on the eyes and pigmentation of normal and hypophysectomized amphibians. Frank H. J. Figge.

The discovery of the Lewises that the indophenol dyes produced a marked pallor of the skin and loss of the pigmented layer of the optic cup in tadpoles was used to study amphibian pigmentation. Phenol indophenol and ortho-cresol indophenol were tested on a series of six kinds of amphibian eggs: normal and hypophysectomized *Amblystoma punctatum*, black and white Mexican axolotl, Necturi, and bullfrog. The hypophysectomies were performed between the Harrison stages 30–37. The animals were placed in the dye solutions (1 : 500,000 and 1 : 1,000,000) near the hatching stages and kept there continuously for a period of three months. As the animals grew, it was necessary to increase the concentrations. Dye solutions were changed daily, and during the last half of the experiment, twice daily.

From a study of the relative sensitivity of the salamanders to the dye, it was evident that the degree of sensitivity paralleled the metabolic rate. The higher the metabolic rate, the less sensitive the animal, and less effective the dye. The dyes eliminated the pigment from the tails of hypophysectomized *Amblystoma punctatum* larvae, proving conclusively that the dyes do not exert their influence on the melanophores through any intermediate effect on the hypophysis or its products. The white axolotls decolorized the dye as rapidly as the black. The black animals became as white as the white axolotls. The rate of decolorization of the dye was, therefore, not related to the rate of pigment destruction. The dyes were most effective in eliminating the pigment from *Necturus*, the form closely resembling cave salamanders that exhibit pigment and eye defects similar to those produced by the dyes.

The animals raised in dye solutions exhibit eye defects that vary in degree and uniformity with the species, within the species, and even in the same individual. These defects involve not only the retina and pigmented layer of the eye, but also the scleral cartilage, lens and cornea.

The elimination of neutral red by the kidney tubules. Robert Chambers.

August 18

Quantitative studies on blood clotting. H. P. Smith and E. D. Warner.

The basis of the principle of the master reaction in biology. Alan C. Burton.

The principle that the rate of a chain of processes is determined dominantly by the speed of the slowest process in the chain underlies the widely used method of "temperature analysis" of complex biological processes. Its origin in the analysis of a chain of two irreversible monomolecular reactions is traced. A table is calculated showing how much one velocity constant must be slower than the other in this system in order that the slower reaction is within a given percentage of complete dominance of the chain. This requirement is so exacting that when the Arrhenius law of rate of change with temperature is followed, it is impossible that one reaction may be within 10 per cent of a true master reaction at 0° C. and the other similarly dominant at 40° C. unless the "critical increments," μ, differ by at least 16,500 Calories. Smaller temperature ranges require proportionately greater differences in the critical increments. The straightness of a line through observed points on an Arrhenius plot may result from dominance shared by a number of reactions rather than of a single master reaction.

In longer, more complicated chains the requirements for a true master reaction are more exacting, and for systems in the "steady state" the principle has no application.

It is concluded that the principle of the master reaction has a very restricted application even in these idealised chains and still less to the real systems of biology.

Efficiency of photosynthesis in purple bacteria. C. S. French.

Streptococcus varians assimilates carbon dioxide with hydrogen in the light but not in the dark. The effect of hydrogen pressure, pH, wave length, intensity, and temperature on the rate of the reaction is described. The curves of rate against intensity are S-shaped and influenced by the previous treatment of the bacteria.

Using light of λ 852 with λ 894 from a caesium tube the quantum yield of a thin suspension was measured. About four quanta are needed per carbon dioxide molecule reduced, thus pointing to a similarity with green plant photosynthesis in spite of the fact that the net result of the assimilation does not involve an increase of bound energy.

Some effects of ultraviolet radiation on bacteria. Alexander Hollaender. (Much of this material has already been published—*see Jour. Gen. Physiol.*, May 20, 1936 and *Proc. Soc. Exper. Biol. and Med.*, 1935, vol. 33, and a part of the data will be published in collaboration with Dr. Duggar shortly. A monograph containing some phases of the work is in print.)

Myelination in the central nervous system of the albino rat, treated with thymus extract. (Microscopic demonstration.) Albert C. Buckley.

August 25

Cellular behavior in abnormal growths produced by irradiation of grasshopper embryos. E. Eleanor Carothers.

Embryos of *Melanoplus differentialis*, ranging in age from five to nine days, received either 250 or 300*r* units in a single exposure to X-rays. The treated eggs and controls from the same lot were kept at 25° C. and embryos from each group were fixed daily until the eggs were well into diapause. Roughly 50 per cent of the embryos from the treated eggs show abnormal growths which are duplications and misplacements of normal parts. The majority of these structures occur in the head region. Sections of embryos 24 hours after irradiation show cells with pycnotic nuclei scattered in groups throughout the germ band. These pycnotic nuclei are formed by the coalescing of the chromatin at the end of the anaphase to form a homogeneous, basiphilic globule. Such cells are either phagocytized by other tissue cells, absorbed in situ or cast out of the embryo. Forty-eight hours after irradiation some metaphases show fragmented and lagging chromosomes. The most striking feature at this stage is another type of degeneration. The cytoplasm cytolyzes beginning at the periphery. The spongioplasm remains resistant for a time. The nuclear membrane disappears but the chromatin remains normal in appearance. Gradually it too goes into solution without clumping. By the fifth day after irradiation cytolytic lesions have been formed. The cytolysis may be due to (1) injury to the cytoplasm by the X-rays (2) failure or destruction of some other group of cells upon which the cytolyzed cells are normally dependent (3) the phagocytizing of pycnotic cells which transmit a toxic effect. Conclusion: The abnormal growths seem to be the product of groups of normal, healthy cells which either by cytolytic

or pycnotic lesions are separated from their normal association and proceed to form as much of a new embryo as their cells retain the potentiality to develop. The original pycnosis is clearly traceable to definite stages of mitosis at the time of treatment.

The behavior of the cell surface during cleavage. Katsuma Dan, T. Yanagita and M. Sugiyama.

To find out whether the increase in cell surface which must accompany cleavage occurs uniformly over the egg surface or whether it is different in different regions, particles of kaolin were stuck to the naked eggs of the sand dollar *Astriclypeus*. During subsequent segmentation the positions of these particles were recorded by camera lucida outline drawings and photomicrographs. The data obtained by this method represent linear expansion.

As elongation of the cleaving egg begins, the surface near the spindle poles expands, reaches a maximum (ca. 20 per cent increase) about halfway through cleavage, then shrinks somewhat, arriving at a stable value about 5 per cent greater than the initial reading. On the other hand, measurements taken simultaneously in the region of the cleavage furrow show first a slight shrinkage (ca. 20 per cent), then a very large increase (ca. 150 per cent) occurring later than that at the poles (at completion of cleavage furrow) and finally a shrinkage to a stable value which is again much higher than that at the poles (ca. 80 per cent increase over initial reading). The egg surface between these extremes shows intermediate behavior both in magnitude of the maximum increase and retardation of its appearance.

*Modified sexual photoperiodicity in ferrets, raccoons and quail.** T. H. Bissonnette.

In the work with raccoons (*Procyon lotor* (L.)), about fifty controls were used of both sexes, whose behavior and times of pseudo-hibernation and breeding were normal. Three groups (each of one ♂, one breeding ♀, previously successful, and one unsuccessful) were lighted by 60-watt bulbs from October 10, one hour per night for a week, increased by an hour each ten days to eight hours per night from December 10 onward. All had usual care and identical climatic conditions outdoors with temperature from above freezing to − 20° F., and deep snow occasionally. Some ♀ ♀ were removed from lighted cages when they became pugnacious toward the end of gestation or pseudo-pregnant periods; others were not; but without affecting pregnancies or suckling.

Lighted animals did not decrease food consumption nor undergo pseudo-hibernation with onset of cold weather in December and early January as did controls. All females in lighted pens mated from December 16, 19, and 23, respectively, for ten or more days; controls only after February 1 or even April 15 (38–40 days later) with first litters on April 6 and 7 as in the previous year. The three previously sterile ♀ ♀ again failed to have litters; but the productive ones gave litters of four on February 27, one on March 4, and three on March 10, respectively (exact numbers of preceding "normal" season), and suckled and weaned them through sub-zero weather. The former, returned to lighting and males on March 20, again mated without conceiving; of the latter, returned to lighting and males on May 18 and 19, after suckling, two mated from May 24 onward, the other probably did so unobserved; but no young resulted from these second matings. Second mating periods did not occur with controls nor in these ♀ ♀ in their previous "normal" season. Male and female cycles synchronized.

* The data on ferrets will appear in the *American Naturalist* shortly. The data on quail will appear in the October number of *Bird Banding* and need not be gone into here.

Asexual reproduction in Dodecaceria fimbriatus. Earl A. Martin.

Dodecaceria fimbriatus reproduces sexually by pelagic epitokes and asexually by autotomizing single segments in the middle region of the body. This worm lives in a flask-shaped burrow in which it is folded on itself so that the dorsal surfaces are apposed. The fold is located immediately posterior to the stomach in the intestinal region. When a worm is removed from its burrow in the calcareous materials in which it lives, and covered with loose sand or powdered limestone, it folds on itself and forms a burrow similar to those in the normal habitat. This shows that the folding is a fundamental characteristic which must be considered in relation to the morphology and physiology of the worm. When considered from this point of view, the anterior limb of the fold consists of the feeding and digestive mechanisms, while the folded region and the posterior limb, in addition to conveying the faeces to the anus, serves primarily for the storage of reserve food in the cœlome. Single segments are autotomized in this part of the body.

All autotomized units of the worm regenerate. The anterior part regenerates a new posterior end and the posterior part regenerates a new anterior end. A single segment regenerates from eight to twelve segments anteriorly and about thirteen posteriorly. From this stage growth may continue until a complete worm is formed or the anterior and posterior ends may autotomize at the junction with the original segment each of these autotomized parts then forming complete worms.

The physiology of the stomatogastric system in Arenicola marina. G. P. Wells.*

A series of lugworms (*Arenicola marina*) were watched in U-tubes of sea water. The majority showed an activity cycle, periods of rhythmic head movements or of rhythmic extrusion and withdrawal of the proboscis alternating with periods of rest. The duration of a single cycle is somewhat variable, but is commonly six or seven minutes. Experiments on worms dissected in various ways show that the pacemaker determining this phenomenon is a diffuse structure, probably a nerve-net, in the wall of the œsophagus. From this structure, periodic outbursts of excitation travel forward by way of the proboscis to the central nervous system, evoking outbursts of rhythmic activity in the muscles of the proboscis and anterior body wall. Adrenaline excites the œsophageal pacemaker, the rhythmic discharge becoming continuous instead of intermittent. Injected into intact worms, adrenaline causes prolonged, continuous burrowing. The work was done in England, at Plymouth and London, and a detailed report will appear shortly.

General Scientific Meeting

August 27

The effect of lack of oxygen on the permeability of the egg of Arbacia punctulata. F. R. Hunter and E. N. Harvey.

Fertilized and unfertilized eggs were studied in the presence and absence of oxygen under the following conditions: (1) when placed in hypotonic sea water, (2) in hypertonic sea water, (3) in a 0.475 molar ethylene glycol solution in 50 per cent sea water, and (4) in a 0.5 molar ethylene glycol solution in 100 per cent sea water. Eggs which were shrunk in 200 per cent sea water under anaerobic conditions showed a peculiar cortical change. Under the other conditions studied, lack of oxygen had no effect on the permeability of these eggs. These results are in conflict with the slight effect of anaerobic conditions on permeability which had previously

* University College, London.

been reported by Keckwick and Harvey (*Jour. Cell. and Comp. Physiol.*, **5**: 43–51, 1934) and Hunter and Harvey (BIOL. BULL., **69**: 344, 1935). It is suggested that the previous conclusions were due to incorrect interpretation of the data.

A photronic cell was used in measuring the volume changes in contrast to direct measurements of eggs as had been made during the preceding summer. In this way several hundred thousand eggs could be measured at one time, whereas in the previous experiments only a few cells were measured.

Comparative permeability to water and certain solutes of the egg cells of three marine invertebrates (*Arbacia*, *Cumingia* and *Chætopterus*). B. Lucké, R. Ricca and H. K. Hartline.

Permeability of the cell to water is defined as the amount of water that passes per minute through one square micron of cell surface with a difference in osmotic pressure of one atmosphere. This quantity has been determined for the egg cells of the mollusk *Cumingia tellenoides*, the annelid *Chaetopterus pergamentaceus* and the echinoderm *Arbacia punctulata* by measuring the volume changes of these cells in hypotonic sea water by means of a diffraction method.

The course of osmotic swelling of the three kinds of cells is found to be satisfactorily described by the equation

$$\frac{dV}{dt} = K \cdot S \cdot (P - P_{ex}),$$

where dV/dt is the rate of volume change in cubic micra per minute, S the cell surface, $P - P_{ex}$ the difference in osmotic pressure between cell and medium at time, t, and K is defined as the permeability of the cell to water. For *Arbacia* eggs the values of permeability range around 0.1 at 22° C.; the eggs of *Cumingia* and of *Chætopterus* have much higher values which range from 0.4 to 0.5 cubic micra per minute, per square micron of cell surface, per atmosphere.

Permeability to the rapidly penetrating solute ethylene glycol and to the slowly penetrating solute glycerol is investigated by means of a method devised by Jacobs and Stewart. The cells are placed in a medium made hypertonic by dissolving a quantity of the penetrating substance in sea water. In this hypertonic solution the cells at first shrink, but as the solute becomes distributed between the cells and the medium they gradually return to their original size. From the course of such volume changes permeability to the solute is computed. The values are expressed as the number of mols times 10^{-15} which pass per minute through one square micron of cell surface with a concentration difference of one mol per liter.

Values of permeability to ethylene glycol are found to average approximately 4.0 for *Arbacia* eggs; for *Cumingia* and for *Chætopterus* cells the values are approximately 16.0 at 22° C. Glycerol penetrates very slowly into *Arbacia* eggs; the average value for permeability is 0.03; *Chætopterus* eggs have a much higher value which ranges around 6.0.

It is concluded that the egg cells of *Cumingia* and of *Chætopterus* are several times more permeable to water, and to ethylene glycol and glycerol than are the eggs of *Arbacia*.

Permeability of Ameba proteus to ions. S. A. Corson.

A kinetic method of studying surface forces in the egg of Arbacia. F. J. M. Sichel and A. C. Burton.

Estimates of the surface forces of cells have in the past been made by static methods. The procedure used in the present study is a kinetic one and therefore of value although perhaps less accurate.

The measurements were made from a motion picture taken by Dr. Robert Chambers for the purpose of studying the nature of cleavage in the *Arbacia* eggs from which all extraneous membranes had been removed after fertilization. The pictures were made available to us through the courtesy of Dr. Chambers to investigate the part of the problem under discussion.

If, just prior to the completion of the first cleavage, one of the blastomeres be ruptured with a microneedle, its contents are rapidly discharged into the surrounding sea water. If this be done at the proper stage of development, the contents of the remaining blastomere will be discharged through the connecting stalk. This discharge is due to an excess internal pressure which is in equilibrium with the surface forces.

The rate of discharge follows a law which can be explained by elastic forces rather than by surface tension. Assuming Poiseuille's law and Heilbrunn's value for the viscosity at the stage of development under consideration, we arrive at maximum values of 62 dynes per cm^2. for the excess internal pressure and 0.09 dynes per cm. for the tangential surface forces. These are of the same order of magnitude as the determinations of Harvey and of Cole for the unfertilized egg.

Experimental studies on the oil wetting property of the plasma membrane. R. Chambers.

An oil droplet when applied to the surface of the protoplasm of various marine eggs in sea water either adheres to the egg, the surface of contact of the oil becoming flattened, or slips through the protoplasmic surface and occupies a position in the interior with a flattened surface of contact on the inner side of the protoplasmic surface. At least two conditions are necessary for this to occur: (1) the surface of the egg protoplasm must be freed of extraneous coatings which harden in the presence of calcium, (2) the surface of the oil on being brought into contact with the egg, must be expanding in order to be clean. The animal material used were exovates of unfertilized starfish eggs and intact sea urchin (*Arbacia*) eggs carefully shaken and rinsed in calcium-free sea water and then returned to sea water. Olive oil was used. The results obtained indicate that an increase in acidity of the medium to pH 4.0 greatly enhances the slipping of the oil into the protoplasm while an increase in alkalinity to pH 9.5 decreases the effect.

Interfacial films between oil and cytoplasm. M. J. Kopac.

Minute drops (diameter, 10 to 20 μ) of percomorph liver oil injected into immature oöcytes of *Asterias* remain perfectly spherical. This condition holds for any animal, vegetal or mineral oil or non-toxic water-immiscible liquid. The injected oil drop may be readily deformed by compressing or stretching the cell, and on releasing the pressure quickly returns to a spherical shape. Upon cytolysis of the oöcyte, the oil drop becomes heavily wrinkled and considerably deformed. This wrinkling occurs in oöcytes immersed in acidified sea water (pH = 4), in sea water, or in isotonic $CaCl_2$ alkalinized to pH > 9.5. In isotonic $CaCl_2$ acidified to pH < 3.5, wrinkling occurs but never heavily enough to produce a violent deformation of the drop. The wrinkles on the surface and the collapse of the oil drop are due to the formation of a plastic solid film at the interface (cf. *inter alia*, Wilson and Fries. *Coll. Symp. Monog.*, 1: 145 (1923)). In order to get wrinkling and deformation of the oil drop, the oöcyte must be cytolyzed within 2 minutes after the oil is injected. If an oil drop is kept inside an intact oöcyte for 5 to 10 minutes, no wrinkling or deformation will occur after the cell is cytolyzed. Apparently the surface of the oil has changed sufficiently to prevent the formation of a plastic solid film after exposure to the cytolytic products of the cell. The wrinkled oil drop may be readily impaled with a microneedle and removed from the cytolyzed area. The drop behaves as a plastic solid and may be cut into several fragments. Some of these fragments

remain irregular while others become spherical as in ordinary oil drops. This behavior indicates that the interfacial film is a plastic solid which remains in place and does not affect the interior of the oil drop.

The question of recovery from X-ray effects in Arbacia sperm. P. S. Henshaw.

In our early experiments it was found that when mature *Arbacia punctulata* eggs are exposed to X-rays before fertilization the onset of cleavage following fertilization is delayed, the amount of delay varying with two known factors. The first of these is the quantity of radiation administered and the second is the amount of time allowed between treatment and the moment of insemination. The irradiation effect (i.e. cleavage delay) is increased as the amount of radiation applied is increased and reduced as the time between treatment and insemination is increased. The reduction or loss of effect may be regarded as a form of recovery, that is, as observed by determining cleavage time. The recovery process, whatever its nature, is in progress as soon as any irradiation effect is produced (even while treatment is going on), and the rate at which it takes place following treatment is practically exponential with time.

More recently it has been shown that cleavage delay may be produced by irradiating sperm alone and that the effect is to some extent accumulative when both gametes are treated. In practically all cases, however, the effect for equal exposures was found to be greater on the sperm than on the eggs.

During the present season, a series of preliminary experiments have been carried out to determine whether recovery takes place in sperm the same as in eggs, but without exception none has been observed. Thus, since the sperm cell is composed largely of nuclear material, these experiments suggest that the X-ray effect was produced mainly in the nucleus and that the cytoplasm is involved in the recovery process. Lack of recovery in the sperm will probably account for its greater susceptibility.

The respiratory effects exerted by certain organic compounds in relation to their molecular structure. Anna K. Keltch, G. H. A. Clowes and M. E. Krahl.

Previous work by the present authors has shown that nitro and dinitrophenols can, in extremely low concentrations, produce a large increase in oxygen consumption and a complete and reversible block to the division of marine eggs. Phenols containing nitroso or amino groups in place of the nitro lacked these effects. Nitro and dinitrobenzene derivatives having the OH replaced by NH_2, Cl, Br, I, CH_2OH, NR_2, OR, COOH, or $CONH_2$ also lacked these effects. From such experiments it was tentatively concluded that the effects of the nitrophenols on cell respiration and cell division were not due to a reversible oxidation or reduction of the nitro groups.

The present experiments provide an extension and confirmation of this view. Phenols containing two or three halogen substituents in the benzene ring produced respiratory stimulation and division block in fertilized *Arbacia* eggs in the same way and to approximately the same degree as paranitrophenol, dinitrocresol and 2, 4 dinitrophenol. Phenols having only one halogen substituent and no other groups were inactive.

The isomeric hydroxy benzoic acids, the isomeric hydroxy benzaldehydes, and phenols having one aldehyde and one halogen substituent were inactive.

To produce a substantial respiratory stimulation and an accompanying reversible division block in marine eggs it seems necessary to have a phenol hydroxyl group, and a suitable number and arrangement of nitro groups or halogen groups or both. Even replacement of the H of the hydroxyl group by either CH_3 or C_2H_5 rendered the phenolic compounds inactive.

Action of metabolic stimulants and depressants on cell division at varying carbon dioxide tensions. M. E. Krahl, G. H. A. Clowes and J. F. Taylor.

From previous experiments it was not known whether the division block produced in marine eggs by dinitrophenols was attributable to the phenols themselves or to secondary effects of metabolic intermediates or end products, such as an excessive amount of lactic acid produced aerobically.

The following new evidence demonstrates that abnormally large amounts of acidic metabolic products cannot be responsible for the adverse division effects of phenol derivatives: (1) Phenosafranine, an inhibitor of the Pasteur reaction, produced no inhibition of cell division. (2) Penetration of high concentrations of butyric acid produced no division block comparable to that with the phenols. Likewise, concentrations of CO_2 up to 2 per cent produced no marked inhibition of cell division. When either of these penetrating acids was employed in sublethal doses inhibition of division appeared to be equally intense at all points in the mitotic cycle whereas the nitrophenols were more effective before onset of prophase. (3) Division blocking concentrations of the phenol derivatives did not produce an abnormally high aerobic glycolysis in *Arbacia* eggs.

The division block produced in *Arbacia* eggs by a fixed concentration of dinitrocresol was dependent on egg concentration, a greater blocking being produced as the number of eggs per unit volume was increased. This same dependence on egg concentration was found for the effects of various nitro and halophenols on *Arbacia* and for the effect of dinitrocresol on *Chaetopterus*. These effects are just the reverse of those observed when anesthetics are employed to block division. The action of high egg concentrations with nitro and halophenols was proved to be due to the accumulation of respiratory carbon dioxide.

Experiments with added CO_2 showed: (1) A CO_2 tension, which alone had a relatively slight adverse effect on division, intensified the dinitrocresol division block and the magnitude of this effect was more than the sum of the separate effects for the individual reagents. (2) Concentrations of CO_2 from .5 per cent to 2 per cent intensified the respiratory stimulating effects of sub-optimum concentrations of dinitrocresol. (3) In a similar way, carbon dioxide intensified the division block and accentuated the respiratory stimulation obtainable with sub-optimum concentrations of paranitrophenol, 2, 4 dinitrophenol and 2, 4 dichlorophenol.

Further studies on the effect of numbers present on the rate of cleavage in Arbacia. W. C. Allee and Gertrude Evans.

Paradoxical osmotic volume changes in erythrocytes. A. K. Parpart and M. H. Jacobs.

Earlier work by the authors (Biological Bulletin, **65**: 513, 1933) indicated that erythrocytes exposed to solutions of non-penetrating non-electrolytes undergo a reversible decrease in their internal osmotic pressure through an exchange of anions from the cell for OH' ions from the solution, with a consequent increase in the proportion of the total base bound by hemoglobin. Direct evidence, however, was lacking that under these conditions reversible volume changes of the cells occur. This evidence has now been supplied, partly by hematokrit measurements and more clearly by the photoelectric method described elsewhere by one of the authors (Parpart). Appropriate experiments show that in solutions of sucrose which from their freezing points would be expected to be isotonic or even slightly hypotonic to the cells there is a rapid decrease in the volume of beef and rat erythrocytes. The addition of small quantities of salts to suspensions of erythrocytes in such sucrose solutions at first causes a further shrinkage of the cells due to the increased osmotic

pressure of the solution, but unless the electrolyte concentration be too great, this shrinkage within a few seconds is followed by a considerable swelling. The optimum concentration of NaCl for producing swelling is about 0.005 M, which is approximately the same as that already found in unpublished experiments to cause the greatest increase in osmotic hemolysis in hypotonic sucrose solutions.

Further studies on specific physiological properties of erythrocytes. M. H. Jacobs, H. N. Glassman and A. K. Parpart.

It was previously observed by one of the authors (Parpart) that the erythrocytes of the ground-hog are remarkably permeable to certain non-electrolytes of relatively high molecular volume such as erythritol, mannitol, xylose, glucose, thiourea, etc. These observations have been confirmed by a further study of the blood of 3 more animals of the same species, and additional determinations have also been made of the permeability of the erythrocytes of the ground-hog and of 6 other species of mammals to a series of 15 ammonium salts. In general, the erythrocytes of the ground-hog have been found to be no more permeable to the ammonium salts studied than are those of the other species, except in the case of ammonium borate to which they seem to show a unique permeability. Experiments designed to give evidence by osmotic means of leakage of salts from the erythrocytes also failed to show a more rapid escape from those of the ground-hog than from those of several other species. Evidently, therefore, a very high permeability to non-electrolytes is not necessarily associated with a high permeability to electrolytes. In the case of the blood of the rat, two apparently specific characters, not found with the other species studied, have been investigated. They are (a) the complete failure of ammonium benzoate to retard hemolysis by ammonium chloride and (b) a very high resistance to hemolysis by distilled water after a previous exposure to a hypertonic salt solution. The blood of the skunk (3 individuals) has also been investigated. In addition to certain other less striking specific properties, it apparently differs from that of the other species studied in undergoing rapid hemolysis in a 4 : 1 mixture of M/6 NaCl and M/6 $NaHCO_3$.

AUGUST 28

Experiments on the contractile substance of muscle fibers. C. C. Speidel.

Retraction caps of injury may be induced readily in the striated muscle fibers of living frog tadpoles, if these are orientated properly and subjected to electrical stimulation of suitable strength. A primary retraction cap appears first at the muscle-tendon junction. It is a coagulation product, or muscle clot, which involves a few or many sarcomeres of the contractile substance. It quickly stabilizes the injured end of the fiber, prevents loss of tension, and thus makes for the preservation of the remainder of the fiber.

Further electrical treatment usually causes the contractile substance to separate from the retraction cap. As it draws back a second cap forms. If the fiber is long enough, third and fourth caps may arise in similar fashion until ultimately the entire fiber has undergone complete retraction. In the case of long fibers, such as those teased from the gastrocnemius muscle of the adult frog, multiple retraction caps (a dozen or more) may be induced successively. If, however, muscle fibers in the tadpole's tail are orientated with their long axes at right angles to the direction of the current, retraction caps do not form.

Retraction caps may be induced on young regenerating fibers any time after the differentiation of cross striæ, but not before. Retraction caps do not form in either smooth muscle or heart muscle.

A wide variety of methods (and reagents) has been employed in investigating the response of the contractile material, which supplements the data from electrical

stimulation. Many of the phenomena have been recorded by means of ciné-photomicrography.

The injection of aqueous solutions, including acetylcholine, into the isolated muscle fiber. Elsa M. Keil and F. J. M. Sichel.

Very small amounts (5,000 μ^3) of distilled water, Ringer's solution, isotonic sucrose or sodium chloride solutions, when injected into, or blown upon the surface of the isolated single muscle fiber (adductor muscles of frog's leg), produce no visible effects apart from a purely mechanical passive disturbance. This has been found to be true only if a clean, previously unused, or freshly washed micropipette is employed. If a larger quantity is injected or if the injection is done too rapidly, or if a previously used pipette is employed, a localized contraction of the interior of the fiber occurs, which is immediately reversible on stopping the injection. Coincident with the contraction the fibrillar structure becomes sharply visible at the tip of the micropipette. The contraction is accompanied by the diminishing of the inter-striæ distances.

The application or injection of $CaCl_2$, as pointed out by Chambers and Hale, causes a violent shortening of the fibrillar material. This shortening is reversible and occurs even when minimal amounts of M/200 solution are injected.

The application or injection of acetylcholine in concentrations ranging from 1 in 10^3 to 1 in 10^{11} has no effect on the isolated fiber, even when eserinized, beyond the effects described above attributable to distilled water, Ringer's solution, etc.

The fiber preparation is capable of giving a twitch response to electrical stimulation although it is incapable of giving a conducted response, and is apparently denervated. Effects of acetylcholine in concentrations within the range here used have been reported in perfused striated muscles. We conclude that such action is not upon the contractile mechanism directly.

The double refraction of smooth muscle. E. Bozler.

The double refraction of smooth muscle and its changes during contraction are studied by a new method. By means of a mica wedge in front of the analyser, fringes are produced, the position of which indicates the double refraction of any part of the muscle. In some preliminary experiments it was found that, on stretching, the double refraction of the muscle (retractor of the snail *Helix pomatia*) increases linearly with the square root of the length. This change cannot be regarded as a photo-elastic effect because no tension is present within the range of lengths used. The effect produced by increasing the length is probably due to the orientation of molecules previously unoriented. It was furthermore found that during an isometric contraction there is no change of double refraction within the limits of the accuracy of the method (1–2 per cent). The contrary result obtained for smooth muscle may be explained by the fact that the contractile segments of the myofibrils are able to shorten even in an isometric contraction. There is no evidence that production of tension in muscle is associated with a change of double refraction.

Some physical and chemical properties of the axis cylinder of the giant axons of the squid, Loligo pealii. F. O. Schmitt, R. S. Bear and J. Z. Young.

Because of their large size the giant axons of the squid are well suited for the investigation of the physical and chemical properties of typical axis cylinder. The birefringence is, as usual, positive with respect to the length of the fiber, in this case being 5×10^{-6}. In alcohol and certain other reagents, the axis cylinder shrinks from the surrounding sheaths to less than 50 per cent of its original diameter and

may be withdrawn as a single strand retaining considerable birefringence. By using the immersion technique this double refraction was shown to be largely of form character, though a considerable amount of intrinsic micellar contributions is present.

The axis cylinder proteins exhibit striking shrinkage and swelling changes of an osmotic type. These properties may be observed even after fixation for hours in formalin and must be considered in connection with histological methods.

By extruding the axis cylinder material into small volumes of water or salt solution it is possible to investigate the protein components of pure axoplasm. It was found that the principal protein of this material corresponds to that extracted by neutral solutions from lobster claw nerves by Schmitt and Bear. The extrudate dissolves almost completely in distilled water, but the solubility is markedly increased by the presence of salts. Though in this respect the protein resembles the globulins, the fact that addition of acetic acid produces a flocculent precipitate, which is again soluble in dilute alkali, suggests instead that it is a nucleoprotein. Certain other chemical evidence obtained in connection with attempts to prepare nucleotides from this protein offer confirmation, but complete proof awaits experiments to be carried out with the more abundant lobster nerve material.

The structure of the eye of Pecten. G. Schoepfle and J. Z. Young.

To assist in Dr. Hartline's investigation of the action potentials in the optic nerve of *Pecten irradians* we have re-examined the structure of the eye, using various general histological and special neurological techniques. As has been shown by Dakin, Küpfer and other workers the retina contains two distinct layers of sensory cells, each giving rise to a nerve. The two nerves run separately for some distance before uniting behind the eye.

In Golgi preparations it is easy to see that the processes of the elongated cells of the proximal layer pass into the nerve without the intervention of any synapse. In the case of the more rounded cells in the distal layer the connections are less easy to make out, but in favorable cases it can be seen that axons arising from the cells pass directly into the nerve which arises from this part of the retina. While it is possible that there are other types of cell present which have not been stained in our Golgi preparations, yet since we have never seen any indication of them it seems probable that both the proximal and the distal layers consist only of primary sense cells and interstitial cells, and that there are no other nerve cells, and no synapses in the eye.

A further point of interest is that the fibers of the common optic nerve, formed by fusion of the branches arising from the two layers, run across the ring nerve which runs around the edge of the mantle, and pass directly into the radial pallial nerves. The optic nerve bundle sometimes runs with the ring nerve for a short distance but certainly the great majority, and probably all of its fibers pass on into one of the pallial nerves without passing around the mantle for any great distance.

The discharge of impulses in the optic nerve fibers of the eye of Pecten irradians. H. K. Hartline.

The discharge of impulses in the optic nerve fibres of the eye of the scallop, *Pecten irradians*, has been studied by recording their amplified action potentials. In the whole optic nerve the response to illuminating the eye is strongest at the beginning of illumination, diminishes distinctly after several seconds, but nevertheless continues as long as the light shines. When the light is turned off there is another strong outburst of nerve impulses, lasting several seconds.

It has been possible to study separately the responses of the two different layers of sensory cells in the *Pecten* eye, since the fibres from each layer form distinct branches of the optic nerve.

Impulses are discharged in the fibres from the proximal sensory cells only when the eye is illuminated. They cease when the light is turned off. Records from single fibres show a regular series of impulses beginning at a high frequency and adapting fairly rapidly to a level which is maintained as long as the light shines. The level of frequency is higher the greater the intensity of illumination.

Impulses are discharged in the fibres from the distal sensory cells in response to cessation of illumination. This discharge may last several seconds, or even minutes; it is abruptly stopped if the eye be re-illuminated. Records from single fibres show that the frequency of the discharge is initially high, but diminishes rapidly. The level of frequency and the duration of the discharge are greater the more intense and more prolonged the preceding illumination. In some cases, following strong illumination, it has been observed that the discharge in these fibres may break up into rhythmic bursts of impulses, the bursts occurring synchronously in all the fibres of this branch of the optic nerve.

The effect of light on the CO-poisoned embryonic Fundulus heart. K. C. Fisher and J. A. Cameron.

When embryos of trout, salmon, or *Fundulus* are subjected to mixtures of CO and O_2 dissolved in water the frequency of the heart falls below the normal value by an amount depending on the ratio of the concentrations of O_2 and CO, and the inhibition is relieved by visible light to an extent depending on the intensity of the illumination. The behavior found suggests that in the presence of CO the frequency of the heart depends on the ratio of the CO-free and CO-combined fractions of Warburg's "atmungsferment." Light causes the dissociation of the CO-ferment complex, more O_2 is then able to pass through the system and the beat frequency recovers. If this interpretation is correct, it should be possible to replace "O_2 consumption" in Warburg's theoretical development by "heart beat frequency." Using the formula $\frac{\text{uninhibited frequency}}{\text{inhibited frequency}} = K\frac{[O^2]}{[CO]}I$ we have partially tested this possibility using *Fundulus* embryos, and, as required by the equation, find that at a fixed $\frac{[O_2]}{[CO]}$ the $\frac{\text{uninhibited}}{\text{inhibited}}$ is a function of I, the light intensity. The slopes of the graphs of $\frac{\text{"uninhibited}}{\text{inhibited}}$ against I lines" vary in the expected direction with changes in $\frac{[O_2]}{[CO]}$, though the data are not yet complete enough to be quantitative in this respect. The time course of the frequency change upon illumination of a CO-poisoned preparation is that of a first order process, which process is probably not the decomposition of the CO-ferment compound.

We conclude that there is a reactant interposed between the "atmungsferment" and the rhythmic production of stimuli to the heart muscle. Change in the light intensity changes the concentration of this reactant as indicated by the frequency of the heart and it is this reactant whose change in concentration is an apparent unimolecular chemical reaction.

Prevention of edema in frog perfusions in the absence of serum proteins. G. Saslow.

Drinker has observed that leakage through frog capillaries can be prevented for over 3 hours by the addition of a minimum of 15% of horse serum to a perfusion liquid such as 3% acacia in 0.65% NaCl. The acacia alone is ineffective, even if enough be used to make the oncotic pressure three times that of frog blood. Drinker concludes that serum possesses a peculiar power of restraining capillary leakage.

In preliminary experiments on this subject, it was observed that the web of the foot of perfused frogs developed no edema in 5 hours (when the experiments were terminated) when either of the following solutions was used:

1. 5% gum acacia
   0.014% KCl
   0.75% NaCl
   P (NaH_2PO_4, Na_2HPO_4), 10 mg./100 cc.
   23% beef red cells (washed twice)
   pH 7.2
2. The same as 1, except that the acacia concentration was 3%.

During perfusion the solutions were continuously stirred with oxygen from a tank.

It is thus clear that if oxygenation be adequate, the serum constituents can be replaced completely by a colloidal polysaccharide in such concentration as to give approximately the same viscosity and oncotic pressure to the fluid. That serum possesses any special power of restraining capillary leakage, under normal circulatory conditions, appears quite improbable. These conclusions are in complete accordance with those already published by Amberson, Stanbury and co-workers for mammals.

Behavior of frog tadpole epidermal cells during seven successive regeneration periods. J. A. Cameron and K. O. Mills.

Bullfrog tadpoles about 5 cm. long were kept at high temperature and exposed to alternate twelve-hour periods of darkness and intense visible light. Every twenty-four hours a 5 mm. piece was cut from the tail of each experimental animal. The plane of the cuts was at right angles to the tail-axis. The seventh cut was usually fatal, but a few lived until the ninth day. Histological study showed that after each injury the epidermis covered the exposed wound surface by migration from the region anterior to the cut. Since on each successive day a new and more extensive injury than that of the day preceding stimulated new migration, it might have been expected that mitosis would increase with the severity and duration of the demand for new cells. In no case did the rate of mitosis in the skin of the regenerating animal equal that of the controls or of the first specimen from the experimental animal concerned. The counts included all the skin of the parts cut off and almost all the mitoses found were in lateral, non-migrating cells. The sample for each day showed a definite decline in the mitotic rate as compared with the rate for the preceding day.

Two explanations are suggested: one, that each injury induced or contributed to the production of an inhibition of mitosis in the skin which extended at least as far as 5 mm. anterior to the cut and lasted for at least twenty-four hours; the other, that cells lost through migration are not in all cases replaced by mitosis of epidermal cells but by addition and differentiation of traveling cells from the connective tissue of the dermis.

Observations on conditions affecting growth of cells and tissues, from microscopic studies on the living animal. E. R. Clark and Eleanor L. Clark.

A careful microscopic study of the growth and differentiation of new tissue in many double-walled transparent chambers installed in rabbits' ears, in which the tissue has been subjected to a variety of experimental conditions, has revealed the following.

The inflammatory exudate which fills the space within the first 24 hours after installation, consisting chiefly of blood plasma, erythrocytes singly or in clumps,

fibrin and leucocytes, all in varying proportions, promotes rapid growth of cells from surrounding tissues. If the chamber is subjected to continued insults, so that there are repeated renewals of the inflammatory exudate, new growth continues until the space is completely occupied by new tissue. If, however, the chamber is protected from insults so that no appreciable renewal of the inflammatory exudate occurs but only the normal interchange between blood and outside intervascular substance, the rate of growth gradually diminishes. Thus in a chamber demonstrated at this session, in which the maximum success has been achieved in eliminating insults, such a slowing-down of growth occurred that, in the central part of the chamber, a lake of semi-fluid material filling more than half the thickness of the space has remained for a month uninvaded by fibroblasts or blood-capillaries.

It seems obvious that, to induce growth of cells there must be present in their immediate environment growth-promoting conditions which involve both physical and chemical properties. The mere presence of a space filled with fluid or semi-fluid material and undoubtedly containing proteins, is not sufficient to induce growth of fibrous or vascular tissue.

Incidentally, the lymphatics which vary in different specimens have been carefully studied in many chambers, and it has been found that the rate of growth of fibroblasts, blood capillaries, etc., shows not the slightest relation to the presence or absence of functional lymphatics.

Some nuclear phenomena in the Trichodina (Protozoa, Ciliata, Peritrichida) from Thyone briareus (Holothuroidea). Laura N. Hunter.

The Trichodina occurring in great numbers in the gut of *Thyone* exists in two forms. Type I inhabits the anterior secretory portion of the gut, type II the posterior absorptive region. Type II individuals are elongated, flattened, possess few pellicular wrinklings and contract seldom. Type I individuals are bag-shaped, possess pellicular annulations as do certain vorticellids and contract frequently. One hundred type I individuals averaged 112 microns long by 75 microns wide as contrasted to 78 by 50 microns in 100 type II individuals. The square root of the average length times width of the corona, a skeletal ring in the adhesive disc, was 51 for 100 type I and 37 for 100 type II individuals. The vegetative macronucleus is elongated and somewhat E-shaped in type I and bean-shaped and compact in type II. In both types it appears vacuolated and heterogeneous,—becoming homogeneous as fission approaches. The resting micronuclei of both forms show striations suggestive of chromosomes. Binary fission occurs similarly in both forms but the early post-fission macronucleus in type I is elongated and bent in contrast to the compact bean-shaped post-fission macronucleus in type II. The micronuclei of both forms divide mitotically, with chromosomes and spindle fibers clearly visible. Association between a large and a small individual also occurs in both types. It is accompanied by macronuclear fragmentation and disintegration and several mitotic divisions of the micronucleus. The exact process is not yet worked out but during the ensuing reorganization the larger individual—in type II at least—contains successively 4 micronuclei and 4 partly granular macronuclear anlagen, then 1 micronucleus and 4 macronuclear anlagen. Cells containing 2 macronuclear anlagen and 1 micronucleus presumably result from a cell division which separates the macronuclei while the micronuclei divide mitotically. The 2 macronucleate stage shows macronuclei free from chromatic granules.

Investigations on determination in the early development of Cerebratulus. S. Hörstadius.

In the 16-cell stage the egg of the nemertine *Cerebratulus lacteus*, which undergoes a spiral cleavage, consists of four layers of each four cells, which layers we may

designate as an_1, an_2, veg_1, and veg_2. Vital staining of the animal half shows that this material gives rise to the ectoderm of the Pilidium-larva, inclusive of a great part of the ciliated band. The vegetative half forms the stomach, oesophagus, the inside of the two lappets, and a part of the ciliated band. veg_2 constitutes the material for the stomach, an_1 corresponds to the greater, most animal part of the ectoderm probably including a small piece of the ciliated band.

Several authors, E. B. Wilson, Zeleny, and Yatsu have found that fragments cleave as fragments, and also, to some extent, shown that they differentiate as fragments. This was confirmed in greater detail. Animal halves have apical organ and ciliated band but no archenteron; vegetative halves have no apical organ, but a large archenteron and a small ectoderm with a ciliated field. $an_2 + veg_1 + veg_2$ has no apical organ and the ectoderm is too small. In $an_1 + an_2 + veg_1$ the stomach is missing, but we find under the ciliated band a wall, corresponding to the oesophagus and the inside of the lappets. an_1 isolated develops into a blastula with an apical organ and a minute piece of ciliated tissue (compare above!), whereas veg_2 isolated dies at a late cleavage stage. $an_2 + veg_1$ differentiates as the middle part of the larva, with a broad ciliated band.

Also when fusing an_1 with veg_2 the fragments develop as they would have done normally: the larva lacks a large ciliated band and oesophagus. The same holds for an animal half transplanted to a meridional half: each component differentiates in accordance with its prospective significance; they seem not to exert any influence upon each other, as is so markedly the case in the sea-urchin egg. The early cleavage stage of *Cerebratulus* thus belongs to the mosaic type of development. But in the uncleaved egg both the animal and the vegetative halves can form complete larvae.

Preliminary evidence as to a source of the growth and the sex-stimulating hormones in the bullfrog. R. Rugh.

Mechanism of hatching in Fundulus heteroclitus. P. B. Armstrong.

Two factors operate to induce hatching in *Fundulus* eggs: (1) the lashing movements of the tail of the embryo, and (2) an enzyme liberated by the developing embryos. The operation of the first of these can be shown by abolishing body movements with chlorotine or KCl at the time when hatching normally should occur. In the absence of the body movements there is no hatching. The operation of the second factor can be shown by treating the eggs immediately after fertilization with 4 per cent to 5 per cent alcohol for the first two days of development. In eggs so treated, some embryos are found with a symmetric menophthalmia and a long, snout-like and sometimes non-patent mouth. In such embryos the hatching enzyme which is produced within the mouth and pharyngeal cavities does not get out into the perivitelline space where it can act on the membrane, and the body movements alone, which appear unimpaired, cannot rupture the chorionic membrane.

The enzyme-bearing cells are seen within both the mouth and pharyngeal cavities. They are unicellular and are full of a granular eosinophilic substance which crowds the nucleus to the periphery of the cell. After hatching, these cells disappear completely.

Hermaphroditism in Mollusca. B. H. Grave and J. Smith.

A study of fifteen species of mollusks has been made during the past summer by the method of paraffin sections, with the object of ascertaining whether hermaphroditism is the rule or the exception among the commonest local species. The selection was made to represent as many families as possible, but those chosen were chiefly lamellibranchs. The Amphineura are represented by *Chaetopleura apiculata*, and the Gastropoda by two marine and two fresh-water species, *Urosalpinx cinereus*, *Nassa obsoleta*, *Planorbis trivolvis*, and *Succinea ovalis*, respectively. Our findings in

the main confirm the generalization by Pelsener (1910) that hermaphroditism reaches its highest expression among the most highly specialized forms, such as the oyster, the scallop, and the shipworm. However, some degree of bisexuality is common, varying from that scarcely detectable, to pronounced hermaphroditism. Some lamellibranchs in which the sexes are quite separate in the adult, have hermaphroditic gonads during the early stages of sex differentiation. Some of the latter are protandric, and some develop from the beginning into males and females, the gonads showing some degree of hermaphroditism. This is especially true of the males. In fact, the testes of young individuals of many species of lamellibranchs contain small and sometimes well developed oögonia as well as mature spermatozoa. The gonads of *Venus mercenaria*, for instance, contain well developed oögonia and spermatozoa, at least during the first year's growth. Loösanoff (1936) describes it as protandric, but the sexes are separate in the adult. Young males of *Chaetopleura apiculata* contain small oögonia which later disappear. Other individuals of this species are females from the beginning of sex differentiation. The sexes appear to be entirely separate in the adult and practically so in the young.

During the breeding season the sexes appear to be separate in the following species, and there is no sign of sex inversion: *Yoldia limatula, Anomia simplex, Ensis directus, Mytilus edulis, Modiolus demissus, Mya arenaria, Cumingia tellinoides, Venus mercenaria*, and *Chaetopleura apiculata*. The young stages of these species have not been studied, with the exception of *Venus mercenaria* and *Chætopleura apiculata*, as described above. In all these forms the sexes are separate in the adults if not throughout life.

Pecten irradians and *Lævicardium mortoni* are truly hermaphroditic, having a functional testis and a functional ovary at the same time. Self-fertilization takes place in this species of *Pecten* as was shown by a series of experiments which resulted in normal development when cross-fertilization was prevented by isolation. Self-fertilization may take place also in *Lævicardium*, although experiments with this animal were not attempted.

There is a change in sex in *Teredo navalis* from male to female, and vice versa. This species is not truly hermaphroditic in that the gonad does not function as an ovary and a testis at the same time but alternately.

In *Planorbis trivolvis* and *Succinea ovalis* an ovatestis is present. The alveoli give rise to both eggs and sperm at the same time. These snails appear to be permanently hermaphroditic. Hickman (1931) gives the breeding season of *Succinea ovalis* as April to September and states that after the hibernating period spermatozoa and eggs develop at the same time, an example of "simultaneous hermaphroditism."

In conclusion it may be said that many species of mollusks appear to be dioecious, but when sexual cycles have been studied adequately, especially during early sex differentiation, some classed as dioecious may be shown to be hermaphroditic or protandric. Sex in many species is not fixed but may be altered both by external and internal changes. The indications are that it may be possible to induce changes in sex in mollusks experimentally.

Papers Read by Title

Separation of the conducting and contractile elements in the retractor muscle of Thyone briareus. H. G. duBuy.

The retractors which are attached to the calcareous ring of the lantern of *Thyone briareus* present a type of muscle which is intermediate between vertebrate striated and vertebrate smooth muscle. The histological characteristics of these retractor muscles and their sensitivity to choline-esters and adrenalin can be compared with properties of vertebrate smooth muscle; their sensitivity to curare and the effect of this drug on the contractile elements can be compared with properties of vertebrate striated muscle.

It can be shown that the action potentials obtained from the nerve-muscle preparation of *Thyone* occur only when the conducting elements are not blocked (for example by curare). After curarisation no action potentials occur while local contractions are still obtainable. These phenomena allow a further differentiation between the properties of the conducting and the contractile elements.

After curarisation of a part of the muscle, the interesting phenomenon of a distal contraction of the non-curarised part can be observed. This is similar to the phenomenon which was first described by Steiman for the single muscle fibers of the frog's tongue. When an electric stimulus is applied to the curarised part of the muscle of *Thyone*, only the non-curarised distal part of the muscle shows a contraction which can be more or less local. After an increase of the intensity of the electric stimulus both the local contraction under the stimulating electrodes in the curarised part, and the distal contraction of the non-curarised part can be obtained. Therefore the threshold for electric stimulation of the conducting elements is markedly lower than the threshold of the contractile elements.

The application of curare prevents the occurrence of spontaneous contractures, etc., which often have hampered investigation of the plastic properties of smooth muscles. Curare does not seem to change these properties. Some differences, however, occur in the extension-curve obtained by means of weights. These differences can be explained by the absence of the activity of the conducting elements.

Hyperparasitism: A species of Hexamita (Protozoa, Mastigophora) found in the reproductive systems of Deropristis inflata (Trematoda) of marine eels. A. V. Hunninen and R. Wichterman.

A species of *Hexamita* was discovered parasitic in the reproductive organs of the trematode, *Deropristis inflata*, found in the marine eel, *Anguilla chryspa*. Of 154 eels examined, 41 were infected with the trematode; 15 of these infected eels harbored 80 trematodes which contained the protozoan parasites.

Measurements showed the length of the protozoön to be 10.2 μ and the width 5.2 μ; the animal being rounded anteriorly and pointed posteriorly. Six flagella originate from the anterior end, being approximately of the same length as the body and directed posteriorly. The two posterior flagella which are approximately twice the length of the body, continue from the axostyles. Two compact club-shaped nuclei are located in the anterior end of the flagellate and extend slightly more than one-third the body length.

The flagellates were limited exclusively to the reproductive organs of the trematode, especially to the female system. They were found in large numbers in the eggs, uterus, oviduct and seminal receptacle. In a few cases they were observed in the vitelline glands and testes. What seemed to be noteworthy was the continued occurrence of the parasites in varying numbers in the eggs of the trematode. In several instances as many as 20 were found in one egg. The protozoa live at the expense of the protoplasmic material of the egg which would normally give rise to a miracidium. In some cases all the eggs in the uterus were parasitized by *Hexamita*, completely preventing miracidial development. The conspicuous absence of *Hexamita* from the intestine of the eels strongly points to a rigid host-parasite specificity between the protozoön and the trematode since flagellates were found in only 2 of the 141 eels examined.

Members of the genus *Hexamita* are of interest because of their widely diversified associations in nature. Some species of the genus are free-living and others associated with vertebrate and invertebrate hosts. This appears to be the first observation of trematodes being parasitized by these flagellates. Further details with figures will be presented in the complete paper.

Cytological studies of the genus Chilodonella. III. The conjugation of Chilodonella labiata, variation? Mary S. MacDougall.

Results of studies of the effects of ultraviolet variation on *Chilodonella* made an investigation of the cytology of this genus desirable. Previous work has shown that the six species whose conjugation and division phenomena have been studied fall naturally into three groups:

1. The Cucullulus group.
2. The Labiata group.
3. The Uncinata group.

Each of these groups has certain definite characteristics peculiar to it, especially as to behavior in conjugation and division.

In Stuart's Pond, a brackish body of water in Woods Hole, Mass., a *Chilodonella* was recently found which looked more like Stokes' figures of *C. caudata* than the fresh-water form of this species. The so-called tail, however, was not constant, and the living material was often confused with *C. uncinata*. The macronucleus is centrally located, however, as it is in *labiata*, and the details of conjugation are identical with that form, i.e. the macronucleus as well as the micronucleus is exchanged during conjugation. There are several points of difference between the new form and *labiata* which seem to be constant. While it is definitely not the typical *labiata* recently described by the author, it is evidently a variation of this type.

It has been found that in nature there is a series of closely intergrading forms in this genus, and further genetic work must wait on more complete knowledge of the cytology of this group.

Comparative hypotonic cytolysis of several types of invertebrate egg cells and the influence of age. Victor Schechter.

The resistance to cytolysis in distilled water was studied for several species of unfertilized invertebrate egg cells. The figures below give the time required for fifty per cent of the cells to cytolyze.

The egg cells of *Mactra solidissima* showed a drop from approximately 8½ minutes when fresh to 3 minutes when 20 hours old. For *Cumingia tellinoides* the corresponding figures were 8 and 3½ minutes. The egg cells of *Heteronereis limbata* showed a resistance of 6½ minutes when fresh and a drop to 4¾ minutes when 28 hours old. For *Echinarachnius* and *Asterias* sufficient quantitative data are not yet available but a similar trend is indicated. Freshly shed *Arbacia* egg cells cytolyze in 3½ minutes.

What significance, if any, lies in the initial difference in resistance of the fresh eggs of these various forms is not yet apparent. In 1932 (Goldforb and Schechter, *Proc. Soc. Exp. Biol. and Med.*) a progressive decrease in resistance to hypotonicity by the ageing egg cells of *Arbacia* was demonstrated. The extension of this finding to other species weights rate of cytolysis as a factor of some importance in the process of ageing. In view of the fact that hypotonic cytolysis is a complicated phenomenon involving the interior of the egg as an osmotic system and also the physical and chemical properties of the cell surface, it is of special interest that all eggs so far studied show a similar trend with age. Preliminary experimental analysis has been made and will be presented in an early paper. It may be of interest to report at this time that, for *Arbacia* egg cells, pretreatment with excess calcium increases resistance to hypotonic cytolysis, decreased calcium lowers resistance and similar solutions also affect the duration of life.

Notes on life cycles of digenetic trematodes. H. W. Stunkard.

Experiments, conducted under carefully controlled conditions, have given the sexually mature stages of two species of larval trematodes from *Nassa obsoleta*. Both species were studied and named by Miller and Northup (1926).

One species, *Cercaria quissetensis*, occurs in about one per cent of the snails examined. The cercariae swim about near the bottom and encyst in various species of mollusks. Experimental infection of *Mya arenaria*, *Mytilus edulis*, *Modiola modiolus*, *Cumingia tellinoides*, *Pecten irradians*, *Ensis americana* and *Crepidula fornicata* was obtained. Metacercariæ recovered three days after encystment were infective for gulls and developed into adults of the species identified by Linton (1928) as *Himasthla elongata* (Mehlis). Constant differences between these specimens and the description of *H. elongata* were observed by Linton and it appears that the worms belong to a distinct species, *Himasthla quissetensis*, first named in the larval stage by Miller and Northup. The specimens found in natural infections are identical with those obtained in experimental ones.

Cercariæum lintoni of Miller and Northup is identical with *Distomum lasium* Leidy, 1891. These larvae encyst in the parapodia of *Nereis virens* and other annelids. After three weeks in the second intermediate host, the metacercariæ were fed to various fishes and developed to sexual maturity in the intestine of the eel, *Anguilla chrysypa*, and the toadfish, *Opsanus tau*. The worms persisted for two to three weeks in the intestine of *Paralichthys dentatus* and *Tautoga onitis*, and it is probable that they reach maturity in these species also. Natural infections were found in the eel. The adult worms are specifically identical with *Zoogonus rubellus* (Olsson, 1868), (synonym, *Zoogonus mirus* Looss, 1901).

Inhibition of gastrulation in Arbacia with Nickel Chloride. A. J. Waterman.

For several summers a study has been made of the effect of various physical and chemical agents upon gastrulation in the sea-urchin, *Arbacia punctulata*. Gastrulation in this form is much more resistant to experimental manipulation than in *Paracentrotus*, while the embryos of the sand-dollar and starfish are particularly sensitive. The chlorides of Na, K, Mg, and Ca alone or in combination, hypotonic and hypertonic sea water, variation in temperature, X-ray and alcohols have been tried.

Recently the blastula and young gastrula stages have been exposed for many hours to various concentrations of some of the metallic chlorides. Of these, certain concentrations of a 1 per cent solution of $NiCl_2$ in sea water have given numerous, excellent exogastrulæ which grew and differentiated a fairly typical body form, skeleton, external gut and stomodæum. The types are not quite as good as those obtained previously with *Paracentrotus* but are the best that have thus far been secured with *Arbacia*. Nickel chloride seems to have a differential effect upon gastrulation in some individuals but the embryos of a culture vary in their susceptibility. Some show total inhibition of any endoderm formation and grow as large, oval vesicles with differentiated ectoderm, apical plate, stomodæum and irregular apron-like growths around the edge of the former vegetal pole region. Nickel chloride also produces skeletal modifications, as well as inhibition and retardation of development depending upon the concentration. A comparison of the effects upon the blastula and young gastrula stages seems to show little or no differential susceptibility previous to or during gastrulation. Gastrulation may be temporarily inhibited for many hours. The general effects upon development, except for exogastrulation, are very similar to those observed with the alcohols and other agents listed above.

Division and conjugation in Nyctotherus cordiformis (Ehr.) Stein (Protozoa, Ciliata) with special reference to the nuclear phenomena. R. Wichterman.

A cytological study of the complete life cycle of *Nyctotherus cordiformis* (Ehr.) Stein, a heterotrichous ciliate from the large intestine of tadpole and adult tree toads, *Hyla versicolor*, was studied. In division, a partial dedifferentiation of parental ingestatory structures occurs, then redifferentiation to be retained by the anterior daughter individual. For the posterior daughter, the ingestatory apparatus arises *de novo*. The macronucleus divides amitotically while the micronucleus divides mitotically, demonstrating small bead-like chromosomes. Dividing animals may be found in tadpole and adult hosts. However, during metamorphosis of the tadpole into adulthood, divisions of the ciliates result in small forms, the pre-conjugants, which are the only ones capable of conjugation. Therefore conjugation takes place only during the metamorphosis of the host. In conjugation, two ciliates fuse along their peristomes. The macronucleus undergoes complete fragmentation while the micronucleus of each conjugant divides three times. The first pregamic division results in two micronuclear products; the second division, in four micronuclear products, three of which degenerate. The remaining product enters into the third pregamic division to produce the two functional migratory and stationary pronuclei. Interchange of migratory pronuclei follows at the fused anterior ends of the conjugants to form the amphinucleus which divides once to produce the micronucleus and the macronuclear anlage. Development and behavior of the unusual macronuclear anlage ("spireme ball" of Stein and Schneider) was observed for the first time.

Ex-conjugants were found nearly exclusively in recently transformed hosts. While the animals remain fused in conjugation for less than a day, the reorganization process of the ex-conjugants takes several weeks. Toward the end of the conjugation process ingestatory structures of each conjugant completely dedifferentiate while a new set arises *de novo* posterior to the old ones.

Conjugation here is interpreted as an effort made by the ciliates to overcome a physiological crisis during the drastic host transformation changes.

Note: The complete paper will appear in the *Journal of Morphology*.

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